Anti-oxidative Role of Cytoglobin in Podocytes and its Association with Chronic Kidney Disease

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Summary

Cytoglobin (Cygb) is a recently discovered member of the mammalian oxygen-binding globin family, in addition to neuroglobin and androglobin and the well-established hemoglobin (Hb) and myoglobin (Mb). Cygb is evolutionary antecedent to Hb and Mb, with which it shares ~30% homology. In contrast to Hb and Mb and, similar to neuroglobin and androglobin, Cygb displays hexa-coordination of the heme iron, implying a role other than oxygen distribution. Cygb is ubiquitously expressed in a wide variety of organs, including heart, brain, liver and kidney, and occurs predominantly in the fibroblast cell lineage, as well as in some neuron populations. Despite extensive research efforts, its physiological role remains unknown, but possible functions include reactive oxygen species (ROS) detoxification and signaling. ROS are detrimental for many diseases, including diabetic nephropathy (DN), a chronic complication of diabetes. Hyperglycemia-induced ROS are responsible for glomerular injuries, such as podocyte apoptosis and/or detachment, which contribute to development of proteinuria.

We aimed to investigate the patho-physiological role of CYGB in the kidney, particularly at the podocyte level, using the human podocyte cell line AB8/13, which expresses abundant endogenous Cygb mRNA levels compared to other kidney-derived cell lines. We established stable CYGB knock-down cell lines using two independent shRNA sequences. These CYGB-deficient podocytes displayed an increase in cell death, as assessed by independent approaches (trypan blue exclusion method, MTT assays, PARP cleavage and TUNEL assays). Moreover, CYGB knock-down cells showed increased accumulation of ROS, as assessed by H_2DCFDA assays and the redox sensitive probe roGFP2-Orp1 at basal levels and upon different stimuli, including antimycin A, H_2O_2 and high glucose treatments. RNA-sequencing based transcriptome analysis of control and Cygb knock-down cells identified dysregulation of multiple genes involved in apoptosis and redox balance. Interestingly, gene array expression analysis of biopsies from chronic kidney disease (CKD) patients showed a pronounced CYGB induction in diabetic nephropathy, validated by RT-qPCR in independent samples. Moreover, genome-wide association studies revealed that CYGB is potentially implicated in CKD. In order to validate our findings *in vivo*, streptozotocin-induced diabetic nephropathy and ischemia/reperfusion kidney injury on a Cygb knock-out mouse model have been initiated.

The identification of CYGB transcript variants with unknown physiological functions added further complexity to our understanding of CYGB regulation. Among five distinct transcript variants, we focused on a transcript that displays an alternative first exon located 10 kb upstream of the canonical one, termed CYGB-A1. In AB8/13 podocytes, CYGB-A1 displayed lower expression

levels compared to CYGB, but appeared to be differentially regulated at transcriptional level, particularly upon oxidative stress.

In conclusion, we demonstrated that Cygb protects podocytes from oxidative stress and cell death. We identified an alternative CYGB transcript variant, regulated by oxidative stress in podocytes and potentially involved in the anti-oxidative function of CYGB. We suggest for the first time the association of CYGB with CKD, particularly with diabetic nephropathy, which is currently under investigation using *in vivo* models. The confirmation of a putative involvement of CYGB will contribute to an improved understanding of how an oxygen-binding globin can take part in the complexity of oxygen and ROS signaling in the diseased kidney.

Zusammenfassung

Cytoglobin (Cygb) gehört zur Proteinfamilie der Sauerstoff-bindenden Globine, welche auch Hämoglobin (Hb), Myoglobin (Mb), Androglobin und Neuroglobin umfasst. Cygb ist ein evolutionärer Vorläufer von Hb und Mb und teilt ~ 30% Homologie mit diesen. Im Gegensatz zu Hb und Mb, und ähnlich wie Neuroglobin und Androglobin, ist das Häm-Eisen in Cygb hexakoordiniert, was auf eine andere Funktion als Sauerstoffverteilung hindeutet. Cygb wird ubiquitär in einer Vielzahl von Organen, einschliesslich Herz, Gehirn, Leber und Niere, exprimiert und tritt vorwiegend in fibroblastischen Zellen, sowie in einigen Neuronenpopulationen auf. Trotz intensiver Forschung ist seine physiologische Rolle bislang unbekannt, aber potentielle Funktionen involvieren die Entfernung reaktiver Sauerstoffspezies (ROS) aus der Zelle und ROS-abhängige Signaltransduktion. ROS sind in vielen Krankheiten, einschliesslich diabetischer Nephropathie (DN), einer chronischen Komplikation von Diabetes, involviert. Hyperglykämie-induzierte ROS führen zur Beschädigung von Glomeruli, wie etwa Apoptose und/oder Ablösung von Podozyten, was zur Entwicklung von Proteinurie beitragen kann. Ziel dieses Projektes war es, die pathophysiologische Rolle von CYGB in der Niere, und insbesondere in Podozyten zu untersuchen. Dafür wurde die humane Podozyten-Zelllinie AB8/13 verwendet, welche im Vergleich mit anderen renalen Zelllinien hohe Spiegel endogener CYGB mRNA exprimiert. Unter Verwendung zweier unabhängiger shRNA Sequenzen haben wir stabile CYGB knock-down Zellen hergestellt. Diese CYGB-defizienten Podozyten haben eine erhöhte Zelltod-Rate, was wir mit unabhängigen Methoden (MTT-Test, PARP-Spaltung und TUNEL-Assay) zeigen konnten. CYGB knock-down Zellen zeigten zudem eine vermehrte Akkumulation von ROS, wie durch H_2DCFDA Assays und mittels der redox-sensitiven Sonde roGFP2-Orp1 sowohl basal als auch auf verschiedene Stimuli, wie Antimycin A, H_2O_2 und erhöhte Glucosekonzentration hin, gezeigt wurde. Durch RNA-Sequenzierung basierte Transkriptom-Analyse von Kontroll- und CYGB knock-down-Zellen konnten wir mehrere Gene identifizieren, welche als Folge des Cygb knock-downs dysreguliert und in Apoptose und Redox-Gleichgewicht involviert sind. Interessanterweise zeigte die Gen Array-Analyse von Biopsien von Patienten mit chronischer Nierenerkrankung (CKD) eine ausgeprägte Induktion von CYGB in Patienten mit diabetischer Nephropathie, was durch RT-qPCR in unabhängigen Proben validiert werden konnte. Darüber hinaus zeigten genomweite Assoziationsstudien, dass CYGB potenziell in der CKD involviert ist. Um diese Ergebnisse *in vivo* zu validieren, wurde die Untersuchung von Cygb knock-out Mäusen in zwei unterschiedlichen Mausmodellen für Nierenschäden, Streptozotocin-induzierter diabetischer Nephropathie und Ischämie/Reperfusion, initiiert.

Die Identifizierung von weiteren CYGB Transkript-Varianten mit unbekannten physiologischen Funktionen erhöht die Vielschichtigkeit der Regulation von CYGB zusätzlich. Wir haben eine von fünf verschiedenen Splice-Varianten näher untersucht. Diese besitzt ein alternatives erstes Exon 10 kb obenhalb des kanonischen ersten Exons und wird als CYGB-A1 bezeichnet wird. In AB8/13 Podozyten zeigt CYGB-A1 im Vergleich zu Cygb eine niedrigere Expression und wird vor allem unter oxidativem Stress unterschiedlich reguliert.

Abschliessend lässt sich sagen, dass CYGB Podozyten vor oxidativem Stress und Zelltod schützt. Wir haben eine alternative Transkript-Variante von CYGB identifiziert, die durch oxidativen Stress in Podozyten reguliert und potenziell an der antioxidativen Funktion von CYGB beteiligt ist. Wir beschreiben zum ersten Mal eine Assoziation von CYGB mit CKD, insbesondere mit diabetischer Nephropathie, welche wir derzeit mit Hilfe von *in vivo* Modellen weiter untersuchen. Eine Bestätigung dieser Beteiligung von CYGB wird unser Wissen und Verständnis von Nierenerkrankungen und der Rolle eines Sauerstoff-bindenden den Globins in der komplexen Regulation der Sauerstoff- und ROS-abhängigen Signaltransduktion erweitern.

1. Introduction

The evolution of the oxygen-dependent metabolism and therefore of complex forms of life required the development of specialized organs and efficient transporting systems for nutrients, oxygen and others gases. At the same time, specific proteins with the capability to reversibly bind oxygen (O_2) evolved to enhance O_2 supply, diffusion and storing. There are three types of respiratory proteins: hemocyanins, hemerythrins and (hemo)globins. Hemocyanins are copper-containing proteins expressed in the hemolymph of many mollusks and arthropods (Burmester, 2002, Markl, 2013). Hemerythrins are binuclear non-heme iron enzymes typical of some protostomes, bacteria and annelids (Bailly et al., 2008, Kurtz, 1999). The most widespread respiratory proteins are (hemo)globins that occur in prokaryotes, fungi (Hoogewijs et al., 2012a), protozoa, plants, invertebrates and vertebrates (Vazquez-Limon et al., 2012, Vinogradov et al., 2013a, Vinogradov et al., 2006, Vinogradov et al., 2005, Weber and Vinogradov, 2001).

1.1 The vertebrate globin family

The vertebrate superfamily of globins counts so far eight members that fulfil an unexpected broad panel of functions not only related to O_2 distribution and delivery.

Hemoglobin (Hb) and myoglobin (Mb) represent the most studied and well-established heme-containing oxygen-binding proteins and for about a century they had been considered the only globin types of vertebrates. Hb and Mb fulfill respiratory functions, supplying the cell with adequate amounts of O_2 for aerobic energy production via the respiratory chain in the mitochondria (Vinogradov and Moens, 2008). Over the last decade, the increasing genomic information has substantially transformed our view of the globin superfamily. Extensive *in silico* searches based on structural information of the globin fold by the groups of Thomas Hankeln (University of Mainz) and Thorsten Burmester (University of Hamburg) lead to the unexpected expansion in vertebrate globins with the discovery of novel globin types. Neuroglobin (Ngb) and cytoglobin (Cygb) perform yet-to-be-illuminated functions and greatly enriched our appreciation of the structural and functional diversity of vertebrate globins (Burmester et al., 2002, Burmester et al., 2000). The identification of additional globin types (GbX, GbE, GbY) with unknown physiological functions and more restricted phyletic distributions has added even further layers of complexity to our understanding of globin gene family evolution (Fuchs et al., 2006, Kugelstadt et al., 2004, Roesner et al., 2005). Recently, a novel family of large, chimeric proteins that contain putative calpain-like and globin-like domains was discovered by comprehensive *in silico* searches in newly available deuterostome sequence data. These chimeric sequences were documented in a phylogenetically diverse array of metazoan taxa, including humans, and in unicellular choanoflagellates and were

named androglobin (Adgb) based on their preferential expression in mammalian testis tissue (Hoogewijs et al., 2012b).

1.1.1 Structure of globins

Globins are metalloproteins of 140-160 amino acid length, able to reversibly bind O_2. They are characterized by a globular domain, named 'globin fold' that surrounds a heme prosthetic group.

Globin fold

Eight α-helical segments, labelled A through H from the N- to the C-terminal, are folded into a characteristic 3-over-3 α-helical sandwich structure named 'globin fold'. Helices A, B, C and E are on the distal side of the heme, whereas helices F, G and H are on the proximal side (Figure 1). Within each helix, the amino acid residues are sequentially numbered, e.g. residue E7 occupies the seventh position within the E helix. In order to keep the uniform labelling, globin amino acid sequences and structures are compared using such defined topological sites as reference (Kapp et al., 1995, Perutz, 1979). Some residues are always conserved among all globins i.e. the proximal HisF8 and PheCD1 (Vinogradov et al., 1992). Highly conserved residues include the distal HisE7, PheCD4 and ProC2.

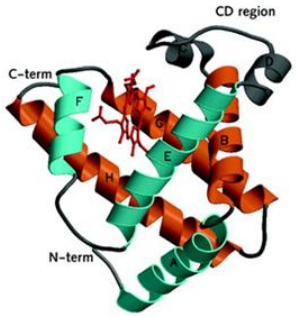

Figure 1. Classical globin fold. A ribbon view of the typical mammalian globin fold is depicted: the 'three-over-three' α-helical sandwich is highlighted in two colors and the helices are labelled according to the conventional globin nomenclature (A-H, starting from the N-terminus). The heme prosthetic group is represented in red (Vinogradov et al., 2005).

Despite the vast variety in the primary amino acid sequence, the 3D structure of the globin fold, and therefore its properties, are highly conserved through the globin superfamily. Globins display a hydrophobic core that surrounds the heme group and hydrophilic surfaces that maintain globin solubility (Lesk and Chothia, 1980). The core hydrophobicity is ensured by 37 conserved non-polar amino acid residues of the globin fold (Gerstein et al., 1994, Vinogradov et al., 2007, Wajcman et al., 2009), summarized in Table 1. The replacement of any of these amino acids alters the hydrophobic/hydrophilic properties of the globin and generates unstable Hb molecules. The most famous mutation of the globin fold is represented by glutamine-to-valine mutation in position 6 of Hb β-chain (B6) that results in sickle Hb (HbS) responsible for sickle cell anemia (Ingram, 1957). The replacement of external hydrophilic amino acid (Glu) by a hydrophobic one (Val)

causes intracellular rearrangements in HbS that aggregates in clumps and compromises erythrocyte shape and function.

Table 1. Conserved residue in the globin structure. A mutation in residue B6 (in bold) generates HbS that is responsible for sickle-cell anemia. Adapted from (Wajcman et al., 2009).

Hydrophobic residues at contacts between helices	A8, A11, A12, A15, **B6**, B9, B10, B13, B14, C4, E4, E7, E8, E11, E12, E15, E18, E19, F1, F4, G5, G8, G11, G12, G13, G15, G16, H7, H8, H11, H12, H15, AND H19,
Inter-helical residues	CD1, CD4 and FG4
Binding to heme iron	HisF8

Heme

The Fe-protoporphirin IX is the prosthetic group of globins, located in the hydrophobic core of the globin fold and noncovalently bound to the globin protein. It derives from porphyrin, a heterocyclic macromolecule consisting of four pyrrolic groups that coordinate a central iron atom. In the Fe-protoporphirin the tetrapyrroles rings externally bind two vinyl groups (positions 2-4), two propionate groups (positions 6-7) and four methyl groups (positions 1-3-5-8).

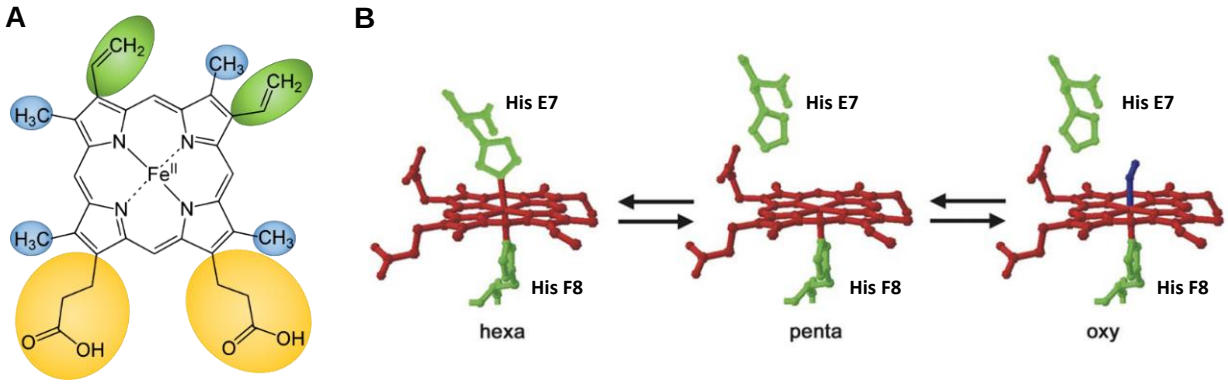

Figure 2. Heme structure. A. Schematic representation of heme, with central Fe^{2+} coordinated by four nitrogen atoms (N) of the pyrrol rings. Two vinyl groups (green), two propionate groups (yellow) and four methyl groups (blue) are bound externally to the pyrrol rings. **B**. Scheme depicting hexa-to-penta-coordinated binding scheme of heme. In the hexa-coordinated configuration, the binding of an exogenous ligand (blue) occurs when it displaces the endogenous distal HisE7, passing through the intermediate state of penta-coordination, where the binding site is free.

The central iron atom can reversibly bind O_2, CO_2 but only in the ferrous state (Fe^{2+}), even though it can display an oxidation state of 3+ (ferric). The Fe coordination number is 6 and it represents the amount of possible binding sites in both ferric or ferrous states. The heme iron is coordinated on four sites by the nitrogen atoms of the pyrrol groups, whereas the two remaining bonding positions are exposed at both edges of the heme plane. One of them is always coordinated by a highly conserved internal proximal histidine residue (HisF8). In the unbound state (e.g. de-oxy) the sixth coordination site of Fe^{2+} can be free (penta-coordinated) or can bind an endogenous ligand (hexa-coordinated) (Figure 2B). Penta-coordination is typical of respiratory globins i.e. Hb and Mb, where the unbound site allows direct interaction with exogenous ligands (e.g. O_2, CO and NO) flattening the iron-heme structure. In the hexa-coordination scheme, typical of all the recently identify globins, the sixth coordination site of iron is bound to an endogenous distal His residue (E7). Therefore, exogenous gaseous ligands (i.e. O_2, CO and NO) must displace the HisE7, leading to changes in protein conformation (de Sanctis et al., 2004b). However, hexa-coordination is not a static structure: Cygb and Ngb are characterized by hexa-to-penta equilibrium that modulates exogenous ligand affinity (de Sanctis et al., 2004b). While no functional interpretation for heme hexa-coordination currently exists, it is thought to point to a role other than classical Hb- and Mb-like oxygen delivery. Moreover, hexa-coordination confers higher stability to changes in temperature compared to penta-coordinated globins (Hamdane et al., 2005, Uzan et al., 2004). Phylogenetic analyses suggest that hexa-coordination is the ancestral binding scheme of heme, whereas penta-coordination emerged later to fulfil respiratory functions.

Heme is not solely restricted to globins as co-factor: it can be associated to different proteins including cytochromes, catalases, heme peroxidase and endothelial nitric oxide synthase oxygenases. In this “non-globin” context, heme is involved in redox reactions and in electron transport chain in mitochondria. Due to the high content of heme, liver, kidney and brown fat display a dark red color.

1.2 Globin evolution

Globins emerged more than 1.5 billion years ago concomitantly with accumulation of atmospheric O_2. As consequence, reactive oxygen species (ROS) were also produced, requiring the evolution of an appropriate defense system, including globins. The first O_2-dependent animals were presumably able to satisfy their O_2 supply by simple diffusion, due to their small size. Therefore, the original globin should have displayed O_2-dependent functions, ROS detoxification abilities or O_2 sensing rather than O_2 distribution (Vinogradov et al., 2013a, Vinogradov et al., 2013b).

Phylogenetic analyses revealed that globins are derived from three distinct lineages. The earliest evolutionary clade includes the hexa-coordinated Ngb, Adgb and GbX, which are expressed in vertebrates and invertebrates (Blank and Burmester, 2012, Burmester et al., 2000, Dröge et al., 2012, Hoogewijs et al., 2012b). Cygb and Hbs of *Agnatha* (jawless fishes) might belong to the same evolutionary clade (Hoffmann et al., 2010) or to two close related clades, in which Cygb diverged first (Blank and Burmester, 2012). The latter hypothesis is supported by the identification of Cygb in the genome of a sea lamprey (jawless fish), indicating that it emerged before *Agnatha* and *Gnathostomata* (vertebrates with jaws) separated, ~550 million years ago (Schwarze et al., 2014). In line with this interpretation, Cygb expression is widespread in different cell types and presents hexa-coordination of the iron, which can be considered ancestral (see above). Moreover, *Agnatha* Hbs, deoxy-Hb, Mb and GbE are all penta-coordinated, suggesting that the conversion from hexa-coordination to penta-coordination occurred after divergence from the Cygb clade.
Furthermore, Mb and GbE are closely related and share similar O_2 binding properties and perhaps similar function (Blank et al., 2011a). Similarly, Hbα and Hbβ belong to the same clade, as confirmed by phylogenetic analysis and genomic data, and represent the most recent globins (Schwarze and Burmester, 2013).
Little is known about GbY and its position in the globin evolutionary tree. According to its similarity to some vertebrate globins, it could be associated to the Hb clade, but its function and heme coordination state are still to be clarified.

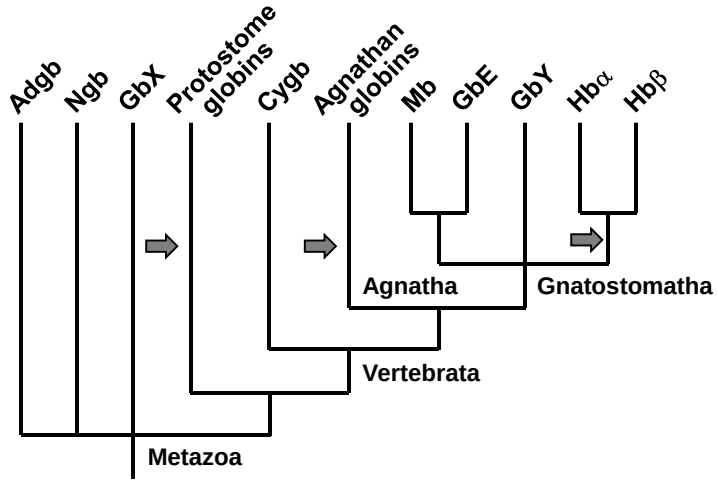

Figure 3. Hypothesized evolution of animal globins. The tree represents a simplified version from (Blank and Burmester, 2012, Hoogewijs et al., 2012b). The O_2 transport function in the circulatory system arose convergently during globin gene evolution (arrows).

1.3 Hemoglobin

Hemoglobin (Hb) is the most abundant protein in erythrocytes, responsible for the red color of oxygenated blood. It reversibly binds and transports O_2 from the respiratory surfaces to the tissue via the circulatory system. Vice versa it can bind CO_2 in the venous blood and carrying it from the tissue to the lungs where it is released (Dickerson and Geis, 1983). Adult Hb is a tetramer consisting of 2α and 2β chains, therefore oxy-Hb carries a total of four O_2 molecules. The Hb-O_2 binding occurs in a cooperative way, meaning that the first O_2 molecule further increases Hb affinity for O_2, facilitating the binding of additional O_2 to the other subunits. This cooperation is graphically represented by the sigmoid shape of the Hb-O_2 dissociation curve (Figure 4), that relates Hb saturation (SO_2) to O_2 partial pressure (pO_2). Different physiological substances and the temperature can modulate Hb conformation leading to a shift of the Hb-O_2 dissociation curve either to the left or to the right, i.e. increasing or decreasing affinity for O_2, respectively. Besides O_2, Hb can bind other substances including H^+, CO_2 and organic phosphate compounds (ATP, GTP or 2,3-diphosphoglycerate (2,3-DPG)) (Berg et al., 2006). This modulation has an important physiological relevance allowing rapid unload/upload of O_2 in specific tissues and in particular conditions. Increases in temperature, pCO_2, and H^+ reduce O_2 affinity (right shift), whereas a decrease in temperature, pCO_2, and H^+ increase O_2 affinity (left shift). The leftward shift is beneficial when the availability of O_2 is reduced, as occurs at extreme altitude, and to upload O_2 in metabolically inactive tissue such as lungs. A rightward shift of the curve is required to release O_2 to metabolically active tissues such as skeletal muscle, or in anemic conditions (Boron and Boulpaep, 2009). 2,3-DPG is produced by erythrocytes and can bind the β-chains of Hb, reducing Hb affinity for O_2 (right shift). Increase in 2,3-DPG facilitates O_2 release from Hb in the peripheral circulation and in hypoxia condition i.e. high altitude. In fish, the major phosphate cofactor is ATP or in some cases guanosine triphosphate (GTP) that generally is a more potent allosteric effector than ATP (Weber et al., 2010). In contrast to other vertebrate hemoglobins, crocodilian Hb exhibit low sensitivity to organic phosphates and high sensitivity to bicarbonate (HCO_3^-), which is believed to augment Hb-O_2 unloading during diving and postprandial alkaline tides when blood HCO_3^- levels and metabolic rates increase (Bauer et al., 1981).

Hb affinity for O_2 could depend also on the expression of different subtypes of α- and β-chains. In humans and in other vertebrate species, different α-like and β-like polypeptides are synthesized at particular stages of development, forming several Hb isoforms specifically expressed during embryonic, fetal and adult life. In humans, the α-like (ζ) and the β-like (γ, δ and ε) globin chains are encoded by specific gene clusters located on chromosome 16 and 11, respectively. For instance, adult Hb is a hetero-tetramer composed by 2α and 2β chains, whereas fetal Hb (HbF)

consists of 2α and 2 γ polypeptides. During pregnancy, maternal bloodstream is the principal source of O_2 for the fetus across the placenta. The γ chains of HbF present higher O_2 affinity compared to adult Hb and are not affected by 2,3-DPG binding (i.e. specific for β-chains), thus facilitating the O_2 uptake from the maternal blood (left shift). HbF is the main isoform during fetal life and is still highly expressed at birth (70-90%), gradually decreasing to 1-2% of the total adult Hb (Wild and Bain, 2004).

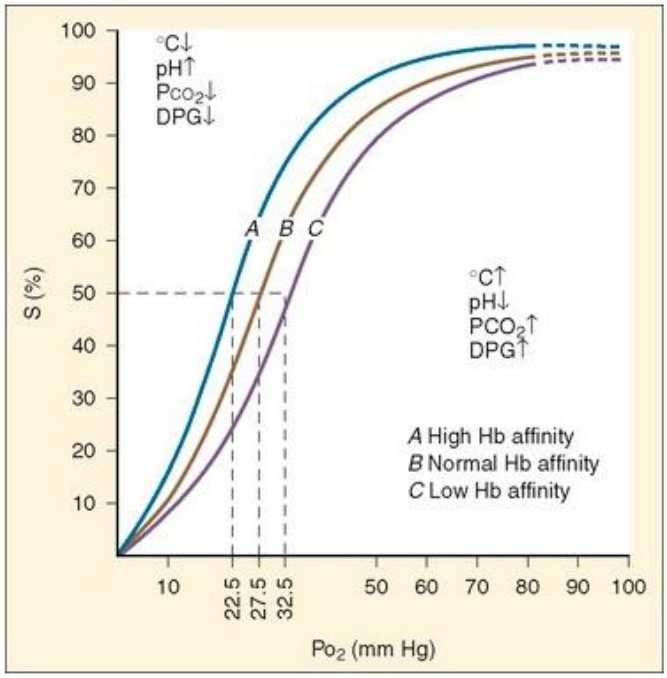

Figure 4. Oxygen-hemoglobin dissociation curve and factors affecting Hb affinity for O_2. Curve *B* is from a normal adult at 37° C, pH 7.40, and pCO_2 35.0 mm Hg. Curves *A* (newborn) and *C* illustrate the effect on the affinity for O_2 of variations in temperature (°C), pH, pCO_2 and 2,3-diphosphoglycerate (DPG). *Hb,* Hemoglobin; *S,* Saturation. Adapted from (Duc, 1971).

When Fe^{2+} is unbound i.e. deoxygenated Hb (deoxy-Hb), the heme group presents a nonplanar "dome" shape and the Fe^{2+} is out of the plane of the porphyrin ring (Figure 5). When the Fe^{2+} binds O_2, the porphyrin ring becomes planar and the heme adopts a flat conformation, allowing the additional binding of O_2 molecules to the other Hb subunits in a cooperative fashion. Due to the presence of numerous aromatic groups, heme is able to absorb and emit UV light at specific wavelengths. The absorption spectra of oxy-Hb and deoxy-Hb are distinctly different, therefore arterial blood appears red (high amount of oxy-Hb) whereas venous blood appears blue (deoxy-Hb). The interaction of gaseous ligands with Fe^{2+} leads to changes in the heme structure as well as in the whole globin. Therefore, several properties, including light absorption and ligand affinity,

are altered upon gas loading. When Fe^{2+} is unbound e.g. deoxy-Hb, the heme group displays a nonplanar "domed" shape and the Fe^{2+} is out of the plane of the porphyrin ring (Figure 5, left). In oxy-Hb, Fe^{2+} binds O_2, the porphyrin ring becomes planar and the heme adopts a flat conformation, changing its light absorption wavelength (Figure 5, right).

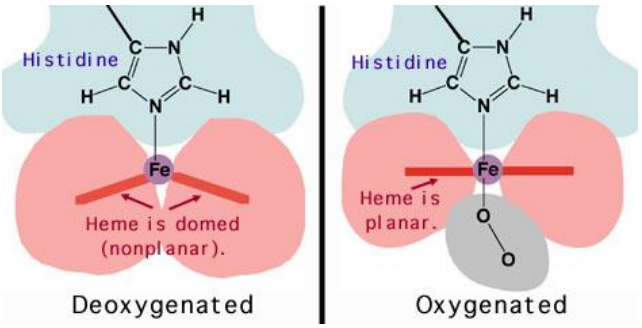

Figure 5. Heme adaptation to ligand binding. In the deoxy-form (left), the central iron atom is pushed out of the heme plane, due to electron-density clouds of the deoxygenated heme group (pink) and the proximal histidine residue (light blue). The binding of O_2 and its electron-density cloud (gray) repulses the heme electron-density clouds and heme adopts a planar configuration (right). Adapted from chemistry.wustl.edu.

Hb is also involved in nitric oxide (NO) metabolism. Briefly, nitrite (NO_2^-), the endocrine reserve of NO, can be converted in NO by deoxy-Hb, due to its nitrite reductase activity. During hypoxia, the increased amount of NO results in blood vessel dilatation (Cosby et al., 2003, Shiva et al., 2011). On the other hand, oxy-Hb can scavenge toxic excess of NO and convert it to nitrate (NO_3^-) (Joshi et al., 2002) with a fast rate.

1.4 Myoglobin

Myoglobin was the first protein to be structurally determined by X-ray crystallography almost 60 years ago (Kendrew et al., 1958). Mb is a monomer consisting of 154 amino acids. Its expression was initially thought to be restricted to skeletal muscle and heart (Wittenberg and Wittenberg, 2003), but further investigation revealed that Mb is also expressed in an unexpected variety of tissues, including smooth muscle (Qiu et al., 1998), endothelial and tumor cells (Gorr et al., 2011) even if in less extent. Mb is upregulated in colon, prostate and breast cancer cells (Flonta et al., 2009, Gorr et al., 2011, Kristiansen et al., 2011) and increased expression correlates with good prognosis of breast cancer patients (Kristiansen et al., 2011). The function of Mb is to extract O_2 from oxy-Hb and distribute it to the mitochondria in metabolically active tissues. For this reason, the half-saturation O_2 partial pressure of Mb (P_{50} = ~1 Torr) is higher than the one of Hb and the Mb-O_2 dissociation curve is represented by a hyperbole. Surprisingly, mice lacking myoglobin are

viable, fertile and show no obvious phenotypic abnormalities, suggesting that Mb is not essential for normal cardiac and skeletal muscle function (Garry et al., 1998). However, using an independent mouse model Gödecke and colleagues revealed that $Mb^{-/-}$ mice activate several compensatory mechanisms i.e. higher capillary density, increase in coronary flow and elevated hematocrit in order to steepen the PO_2 gradient to the mitochondria (Gödecke et al., 1999). Additionally, isoproterenol-induced cardiac hypertrophy in $Mb^{-/-}$ mice and wild-type controls showed a high capacity of $Mb^{-/-}$ mice to adapt to cardiac stress by activating a distinct cardiac gene expression pattern compared to control animals (Molojavyi et al., 2010). Flögel and colleagues suggested an anti-oxidative role of Mb, as hearts from $Mb^{-/-}$ mice were more sensitive to ROS and ischemia injury let to increased ROS production accompanied by a delayed functional and metabolic recovery (Flögel et al., 2004). While some vertebrates completely lack Mb, e.g. frogs (Fuchs et al., 2006, Maeda and Fitch, 1982), some species of icefish and stickleback express non-functional Mb (Hoffmann et al., 2011). To functionally compensate Mb deficiency, frogs might express a monomeric Hb (Maeda and Fitch, 1982) or even Cygb (Xi et al., 2007). Other important functions of Mb are O_2 storage and NO decomposition due to its intrinsic dioxygenase activity (Flögel et al., 2001). Oxy-Mb can oxidize NO to form NO_3^- and ferric myoglobin, protecting the respiratory chain from inhibition of cytochrome-c oxidase by NO (Brunori, 2001). Conversely, deoxy-Mb can drive the opposite effect, reacting with nitrite to form NO, thus inhibiting cytochrome-c oxidase in mitochondria (Hendgen-Cotta et al., 2008, Shiva et al., 2007). Mb can protect also against ROS due to its peroxidase activity (Muzakova et al., 2000). Recently, *in vitro* data indicated that oxy-Mb could interact with fatty acids, providing a potential source of energy for metabolically active tissue (Shih et al., 2014).

1.5 Neuroglobin

Neuroglobin was first identified in 2000 by Burmester and coworkers as a neuron-specific globin. While the other globins display two introns at B12.2 and G7.0, Ngb shows an extra central intron at E11.0 (Burmester et al., 2000). Phylogenetic analysis showed that intron E11.0 is also found in plant globins, whereas animals lost it about 700 million years ago (Hardison, 1996). The presence of intron E11.0 indicates that Ngb has an ancient origin and it diverged earlier than Hb and Mb (Burmester et al., 2000).

Ngb is a monomer, consisting of 151 amino acids. Ngb half-saturation O_2 partial pressure P_{50} = 0.9-2.2 Torr (0.12-0.19 kPa) is comparable to the one of Mb (Burmester et al., 2000, Dewilde et al., 2001). Ngb has a similar structure to Mb, but displays hexa-coordination of the iron atom (Pesce et al., 2003). Ngb is widespread among vertebrates and it is mainly expressed in the

nervous system, from which it derives its name, including the retina, and in some endocrine tissues (Ostojic et al., 2008, Reuss et al., 2002). Its expression pattern in mammalian brain is still a matter of debate (Burmester and Hankeln, 2009, Hankeln et al., 2005) and rigorous quantitative studies of its regional localization are still missing. For this reason, the role of Ngb is largely unknown. At subcellular level, Ngb is localized in mitochondria, therefore it might be involved in oxidative metabolism (Bentmann et al., 2005, Mitz et al., 2009) and O_2 transportation due to its similarity to Mb (Burmester et al., 2004).

Putative roles of Ngb include local or temporal O_2 supply or protection from ROS (Burmester and Hankeln, 2009). As Mb, Ngb could be involved in NO metabolism, acting as NO detoxifier in the oxygenated state (Brunori et al., 2005) or as nitrite reductase when it is deoxygenated (Tiso et al., 2011). Ngb could also prevent apoptosis by reducing cytochrome c release from mitochondria (Fago et al., 2006, Raychaudhuri et al., 2010) and by binding the α subunit of G proteins (Wakasugi et al., 2003, Wakasugi et al., 2011). A putative role in neurite development by altering the PTEN/Akt pathway was recently proposed (Li et al., 2014). Upon hypoxia, Ngb is upregulated in the brain of some animals including zebrafish, the subterranean mole rat Spalax and minke whale (Avivi et al., 2010, Roesner et al., 2008, Schneuer et al., 2012). Its potential function as general neuroprotective protein (Khan et al., 2006, Raida et al., 2013, Sun et al., 2001) is controversial and still a matter of debate (Di Pietro et al., 2014, Kelsen et al., 2008, Schmidt-Kastner et al., 2006). So far, most of the experiments to unravel Ngb functions have been performed *in vitro*.

Ngb-null mice show no morphological changes compared to wild-type mice and no difference in the number of neurons (Hundahl et al., 2011). Pronounced upregulation of c-FOS and HIF1A mRNA was detected upon hypoxia (Hundahl et al., 2011) and behavioral alteration in response to light was observed (Hundahl et al., 2012). In contrast to its putative neuroprotective role, $Ngb^{-/-}$ mice showed a reduction in tissue infarction size upon ischemic injury (Raida et al., 2012). A similar conclusion was provided upon ischemia in mice overexpressing Ngb (Raida et al., 2013).

1.6 Cytoglobin

Cytoglobin (Cygb) is the fourth member of the mammalian globin family, in addition to hemoglobin, myoglobin and neuroglobin. Cygb was independently discovered by three groups (Burmester et al., 2002, Kawada et al., 2001, Trent and Hargrove, 2002). Originally named STAP, that stands for “stellate cell activation-associated protein”, it was renamed “histoglobin” and finally “cytoglobin” due to its ubiquitous expression in many tissues of all vertebrates (Burmester et al., 2002, Kawada et al., 2001, Trent and Hargrove, 2002).

1.6.1 Protein structure of Cygb

Cygb is a 21-kDa protein consisting of 190 amino acids. It contains the typical globin fold (Burmester et al., 2002, de Sanctis et al., 2003), but differently from other globins, it displays additional overhangs of 20 amino acids in length at both C- and N- termini (Burmester et al., 2002) with unknown functional relevance. Crystallography shows that Cygb is a homodimer (de Sanctis et al., 2003, Sugimoto et al., 2004) stabilized by hydrogen bonds, electrostatic interactions (de Sanctis et al., 2004a) and two intermolecular disulfide (S–S) bridges between Cys38(B2) of one monomer and Cys83(E9) of another monomer, and *vice versa* (Sugimoto et al., 2004) (Figure 6). However, Cygb can also be monomeric when heme is at micromolar concentrations, i.e. the range expected for Cygb *in vivo* (Lechauve et al., 2010). In the monomeric form, the two Cys residues might form an intramolecular S–S bond (Hamdane et al., 2003, Lechauve et al., 2010). The state of the cysteines *in vivo* is unknown, but reduction of the intramolecular S–S bonds changes Cygb oxygen affinity in the monomeric form (Hamdane et al., 2003). In both monomeric and dimeric forms of Cygb, Cys38(B2) and Cys83(E9) are crucial for protein stability and might be involved in the ligand binding process (Sugimoto et al., 2004), by promoting conformational changes. A recent study has shown that mutations of the S–S bond in Cygb changed the affinity and rate of binding of exogenous ligands (Tsujino et al., 2014), indicating that the cellular redox state may influence Cygb structure by S-S bond formation or cleavage, thereby affecting O_2 binding (Hamdane et al., 2003). Cygb is a hexa-coordinated protein (Trent and Hargrove, 2002). In the absence of an external ligand, iron is coordinated by six nitrogen molecules – four residing within heme and two derived from two imidazole groups located at the opposite site of the heme plane, His81(E7) and His113(F8) (Figure 6A). This arrangement is similar to Ngb. An exogenous ligand (e.g. oxygen, O_2) must compete with HisE7 for binding to iron. Comparisons between globin primary sequences showed that Cygb shares about 25% homology with Mb and Hb, and 16% with Ngb (Sugimoto et al., 2004). Additionally, Cygb half-saturation O_2 partial pressure (P_{50} = 1 Torr = 0.14 kPa) is similar to the one of Mb (Trent and Hargrove, 2002).

Previous studies using yeast two-hybrid assay and immunoprecipitation/mass spectrometry failed to discover any interactor of Cygb (Hodges et al., 2008). Additionally, Cygb primary sequence lacks any of the known reticulum or membrane retention signals, motifs targeting peroxisomes or RNA, DNA, actin and ribosome binding sequences (Kawada et al., 2001). However, it has been shown that human Cygb binds to lipids, via its transition from hexa- to penta-coordination (Reeder et al., 2011). Latina and colleagues provided evidence for a direct transcriptional regulation of Cygb by p63, a member of the p53 family (Latina et al., 2015).

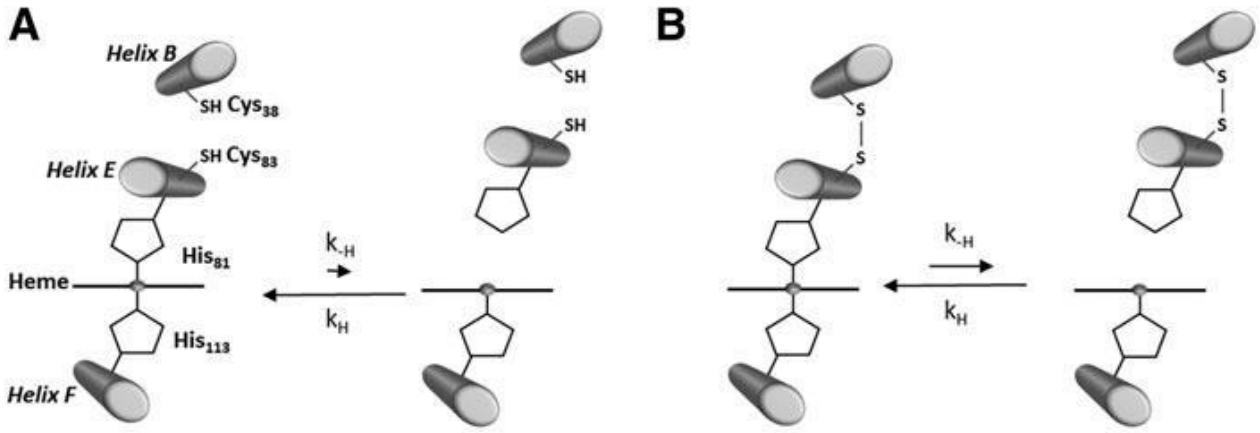

Figure 6. Effect of intramolecular disulfide bond formation on the coordination state of Cygb. A. The heme iron of Cygb is primarily low-spin hexacoordinated bis-His form without the intramolecular disulfide bond between Cys38(B2) and Cys83(E9). **B.** The coordination of the heme iron with the intramolecular disulfide bond is still primarily hexacoordinated, but is shifted partially toward the high-spin pentacoordinate form through increasing the distal histidine dissociation rate (k_{-H}). k_H: association rate. Adapted from (Reeder, 2016).

1.6.2 Tissue and cellular distribution

Cygb is ubiquitously expressed in a broad variety of organs, including heart, liver, kidney, brain, lung, retina and many others (Burmester et al., 2002, Geuens et al., 2003, Hundahl et al., 2010, Mammen et al., 2006, Nakatani et al., 2004, Schmidt et al., 2004, Shigematsu et al., 2008, Trent and Hargrove, 2002). Despite differences at expression levels, Cygb concentration in the tissue is generally low, i.e. in the micromolar range (~1-10 μM). Its localization at tissue level is still a matter of debate. Cygb is mainly expressed in fibroblast-related cell lineages such as hepatic stellate cells (HSCs), chondroblasts, osteoblasts, myofibroblasts (Kawada et al., 2001, Motoyama et al., 2014, Nakatani et al., 2004, Schmidt et al., 2004, Shigematsu et al., 2008). Moreover, Cygb has been found in distinct neuron populations (Geuens et al., 2003, Mammen et al., 2006, Schmidt et al., 2004), hepatocytes (Geuens et al., 2003, Shigematsu et al., 2008), macrophages, muscles (Shigematsu et al., 2008) epithelia, melanocytes (Fujita et al., 2014) and several tumor cells (Emara et al., 2010, Gorr et al., 2011). Cygb localization in the connective tissue and in epithelium explains its ubiquitous expression among different organs.

At subcellular level, Cygb localizes mainly in the cytoplasm of fibroblasts and other mesenchymal cells, epithelium, hepatic cells and connective tissue cells, whereas neurons display predominantly nuclear distribution (Geuens et al., 2003, Hundahl et al., 2010, Ostojic et al., 2006).

Both cytoplasmic and neuronal localization have been observed in different cell types (Emara et al., 2010, Geuens et al., 2003, Shigematsu et al., 2008) and upon Cygb overexpression (Hodges et al., 2008), suggesting that Cygb could translocate to the nucleus. The nuclear localization of Cygb in neurons could speak for a different function in this cell population compared to fibroblasts (Schmidt et al., 2004), but further investigations are needed to confirm this hypothesis. Cygb in the nucleus may contribute to gene regulation, protection from NO and/or reactive nitrogen species (RNS) (see below), particularly relevant in neurons. Cygb nuclear localization has been suggested to occur via passive diffusion, due to its small size and the lack of nuclear translocation motif, but cell-specific factors and specific physiological conditions (e.g. stress) could also promote Cygb compartmentalization in the nucleus (Geuens et al., 2003, Hodges et al., 2008, Schmidt et al., 2004).

Notably, the lack of high quality antibodies against Cygb still represents a limitation to finally solve the distribution pattern of Cygb in specific tissues.

1.6.3 Gene structure

Human *CYGB* is a single copy gene that maps on chromosome 17q25.3. Phylogenetical analysis suggests that *CYGB* emerged during a large-scale duplication event (Burmester et al., 2002). Additionally, *CYGB* gene has the lowest mutation rate among the vertebrate globins, indicating that also large portions of the protein are important for its function (Wystub et al., 2004).

The mammalian *CYGB* gene displays four exons and two introns at position B12.2 (helix B, between nucleotide 2 and 3 in the 12^{th} codon) and G7.0 (before the first nucleotide in the 7^{th} codon of helix G) which are typical for all globins, among vertebrates, invertebrates and plants (Roesner et al., 2005, Stoltzfus and Ford Doolittle, 1993). Genes encoding hexa-coordinated globins present an additional third intron. Although it usually occurs at E11.0 (i.e. Ngb) (Burmester and Hankeln, 2004), the third intron of *CYGB* is uniquely located at HC11.2 (Alberti and Zimmet, 1998, Burmester et al., 2002) (Figure 7).

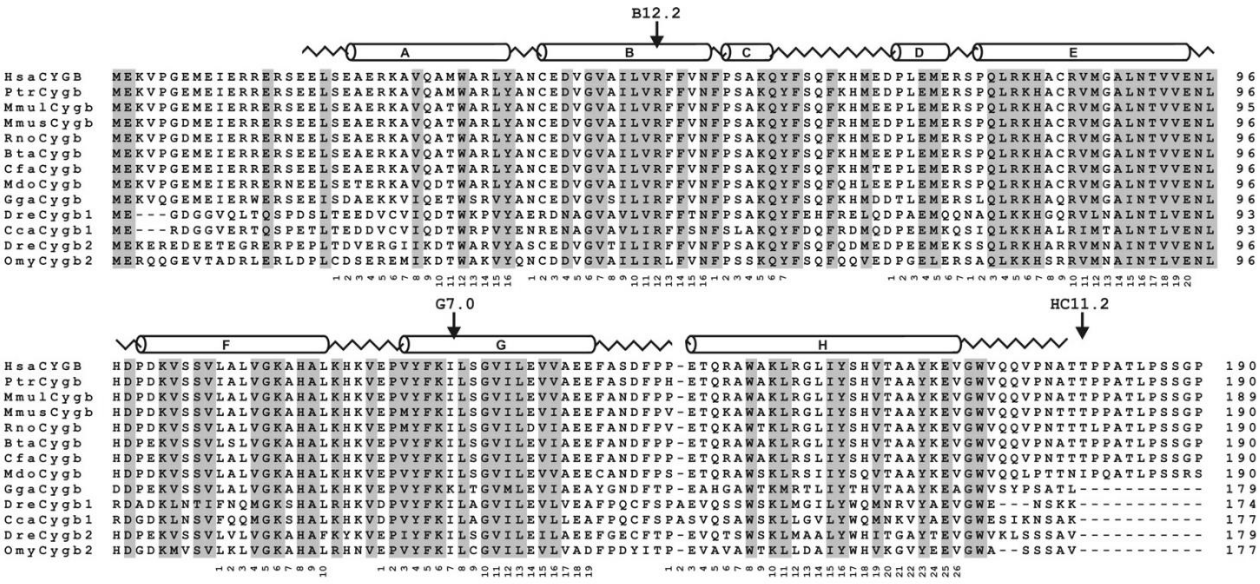

Figure 7. Alignment of cytoglobin amino acid sequences from different species. HasCygb, human (*H. sapiens*); PtrCygb, chimpanzee (*P. troglodytes*); MmulCygb, Rhesus monkey (*M. mulatta*); MmusCygb, mouse (*M. musculus*); RnoCygb, rat (*R. norvegicus*); CfaCygb, dog (*C. familiaris*); BtaCygb, cow (*B. taurus*); MdoCygb, opossum (*M. domestica*); GgaCygb, chicken (*G. gallus*); DreCygb1 and DreCygb2, zebrafish (*D. rerio*); CcaCygb, carp (*Cyprinus carpio*); OmyCygb2, rainbow trout (*O. mykiss*). The secondary structure of human Cygb is shown in the upper row and conserved residues are shaded. α-helices are designated A through H. The black arrows display the position of introns (B12.2 and G7.0 are the common introns in globins, while HC11.2 appeared only in mammalian cytoglobin) (Burmester and Hankeln, 2004).

1.6.4 Gene regulation

Transcriptional regulation analysis of *CYGB* revealed the presence of putative transcription factor-binding sequences on the *CYGB* promoter region (CpG islands), GC-boxes, but no TATA box. Many of the putative transcription factor binding sites are highly conserved in mouse and human, such as hypoxia response element (HRE) and mRNA stabilization sites, suggesting an oxygen-dependent regulation via hypoxia-inducible factors (HIF) (Fordel et al., 2004a). Multiple conserved GC boxes are potential binding sites for stimulatory protein (SP1), activator proteins (AP1, AP2), nuclear factors (NFAT and NF1 families, NF-κB) (Rippe et al., 1999), CCAAT/enhancer binding protein (C/EBP), cellular erythroblastosis virus E26 oncogene homolog 1 (cETS-1) (Guo et al., 2006, Wystub et al., 2004) and Krüppel-like zinc finger proteins (Chen and Davis, 2000), which may be involved in Cygb upregulation during hepatic stellate cells and liver fibrosis. AP1, NF-κB and C/EBP transcription factors could mediate transcriptional activation of different genes involved in inflammation, hypoxia and oxidative stress (Cloutier et al., 2009, Cummins and Taylor, 2005,

Novo and Parola, 2008). The minimal promoter region of *CYGB* gene from -1113 to -10 upstream of the start codon includes one c-Ets-1 and three SP1 binding sites that are critical for Cygb transcriptional activity in normoxia (Guo et al., 2006). NFAT and AP1 can induce Cygb expression, mediating calcineurin signaling (Singh et al., 2009). Moreover, Cygb has been suggested to be a downstream target of tumor growth factor β (TGF-β), platelet-derived growth factor, protein kinase C (Nakatani et al., 2004) and Jun (Norifumi Kawada, O_2BIP meeting 2016) signaling.

1.6.5 Putative functions of Cygb

While Hb and Mb are well-known for their respiratory functions, different roles have been suggested for the newly identify globins, including involvement in NO metabolism, detoxification of ROS, protection from apoptosis, lipid metabolism, cell signaling and hydrogen sulfide (H_2S) turnover (Figure 8) (Burmester and Hankeln, 2009, 2014, Hankeln et al., 2005, Rios-Gonzalez et al., 2014).

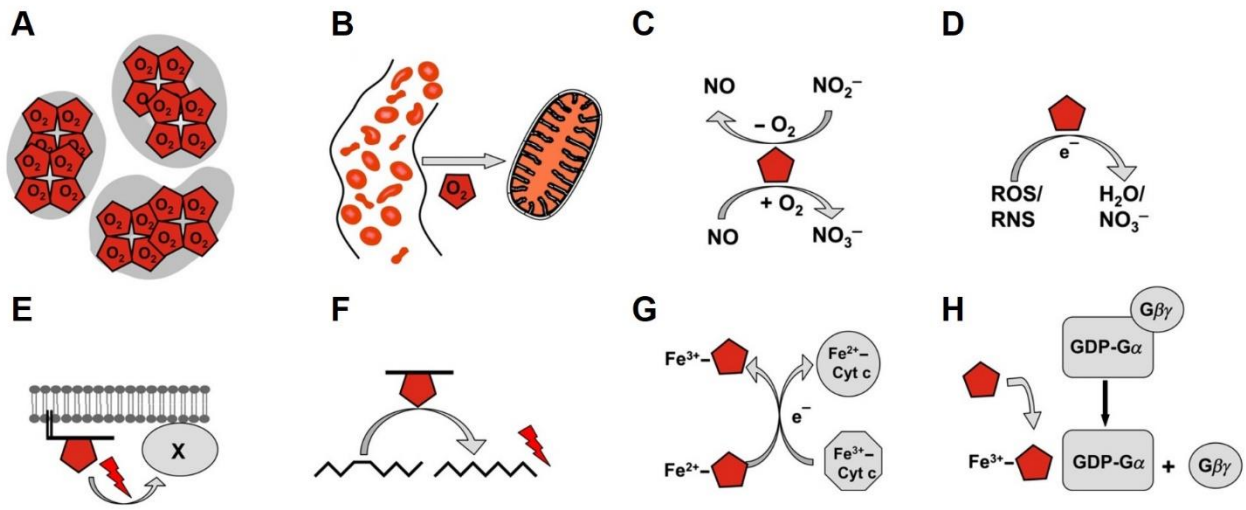

Figure 8. Postulated functions of globins. A. Hb transports O_2 in the blood, **B.** Mb and GbE (and possibly Ngb) enhance intracellular O_2 supply to the mitochondria, **C.** Hb, Mb, Cygb and Ngb may convert NO to NO_3^- at high pO_2 and NO_2^- to NO at low pO_2, **D.** Ngb, Cygb and Mb may detoxify reactive oxygen or nitrogen species, **E.** GbX may be a component of a membrane-mediated signal transduction chain, **F.** Cygb may trigger a lipid-signaling cascade, **G.** Ngb may prevent hypoxia-induced apoptosis via reduction of cytochrome c or **H.** may act as signal protein by inhibiting the dissociation of GDP from Gα. Adapted from (Burmester and Hankeln, 2014).

1.6.5.1 Oxygen storage, transport and sensing

Most of the globins are able to reversibly bind O_2 as well as other gaseous ligands. Due to its homology to Mb and their comparable half-saturation O_2 partial pressures of 1 Torr, Cygb was thought to contribute to the intracellular O_2 supply (Trent and Hargrove, 2002), acting as an O_2 reservoir or as signal transducer in O_2 sensing pathways (Geuens et al., 2003, Hankeln et al., 2005). The alteration in cellular redox state could promote conformational changes in Cygb and increase O_2 release, e.g. via reduction of S-S bridges by reducing agents (H^+, NADH) (Hamdane et al., 2003). As discussed below, Cygb could also contribute to O_2 sensing by modulating NO levels.

1.6.5.2 Cygb in hypoxia

Hypoxia is a (patho-)physiological condition in which cells experience low O_2 tension and therefore the O_2 supply is not adequate. High altitude, intense exercise, poisoning (i.e. drugs, carbon monoxide, cyanide), anemia, tumor proliferation (avascular) and ischemia are several common causes of hypoxia. Mammals adapt to hypoxia at both systemic and cellular level. The systemic response occurs when the whole organism is exposed to low pO_2, as in the case of high altitude, and includes a series of changes involving mainly the respiratory and vascular systems, such as hyperventilation and increase in heart rate. This cellular response requires the activation of oxygen-sensitive pathways within the cell and can be restricted to a specific tissue/group of cells. Hypoxia inducible factors (HIF) play a central role in the cellular adaptation to hypoxia, being part of the “pVHL/PHD/HIF pathway”. In normoxic conditions, the oxygen-sensors PHD (prolyl hydroxylases domain-containing proteins) hydroxylate specific Pro residues on the HIF-α subunit in a O_2-dependent reaction (Kallio et al., 1999). Hydroxylated HIF-α is recognized by an E3 ubiquitin-protein ligase, pVHL (von Hippel-Lindau protein), and targeted for proteasomal degradation (Ohh et al., 2000). In hypoxia, the hydroxylation reaction cannot take place and HIF-α subunits are stabilized, translocate into the nucleus and dimerize with HIF-β, forming the transcriptionally active complex (Schofield and Ratcliffe, 2004). The heterodimer recruits co-factors such as p300/CBP in order to regulate transcription of target genes (Arany et al., 1996). Another member of the hydroxylase enzyme family is the factor inhibiting HIF (FIH), which hydroxylates HIF-α on an asparagine residue, blocking the interaction between HIF-α and p300 (Lando et al., 2002). Cygb expression is increased upon hypoxia at both mRNA and protein level in a wide variety of organs (Fordel et al., 2004b, Fordel et al., 2007a, Mammen et al., 2006, Schmidt et al., 2004), including kidney, eyes, brain, heart, liver, skeletal muscle and in different cellular models (Fordel et al., 2004b, Guo et al., 2007, Singh et al., 2009). The hypoxic

upregulation of Cygb is regulated by HIF-1α and can be reversed by a period of reoxygenation, in which Cygb levels return to baseline (Fordel et al., 2007a). As transcription factor, HIF-1 binds to specific sequences named HREs present in the promoters and regulatory regions of hypoxia-regulated genes. Dual-luciferase assay and site directed mutagenesis showed the presence of three HREs, located at -141, -144 and -448 bp from the transcription start site of the *CYGB* gene, which are essential for Cygb hypoxic activation (Guo et al., 2007). Additionally, HIF-1$\alpha^{+/-}$ haploinsufficient mice subjected to hypoxia display a reduction in Cygb upregulation in brain, liver, muscle and heart, confirming the importance of HIF-1α for Cygb hypoxic regulation (Fordel et al., 2004a). Overexpression of Cygb is associated with increased cell viability *in vitro* (Hodges et al., 2008, Stagner et al., 2009, Yu and Gao, 2013) and *in vivo* (Singh et al., 2014, Xu et al., 2006), suggesting a cytoprotective role of Cygb, particularly under stress conditions. One of the proposed functions of Cygb during hypoxia is that Cygb could help maintaining an aerobic metabolism in the cells while protecting them against ROS (Stagner et al., 2009).

1.6.5.3 Cygb regulates NO metabolism

Overview on NO metabolism

NO is a signaling molecule that exerts several important regulatory functions in both physiological and pathological conditions. Indeed, depending on its concentration and the microenvironment, NO could act as a messenger or cytotoxic molecule (Thomas et al., 2008). NO production is catalyzed by various isoforms of NO synthase (NOS). NOS are dimeric proteins characterized by a C-terminal reductase domain, a N-terminal oxygenase domain associated to a heme group. In humans, there are 3 isoforms of NOS: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). All of them use L-arginine, O_2, and NADPH as substrates to produce aerobic NO and L-citrulline (Forstermann and Sessa, 2012). Due to its high affinity to ferrous heme, NO binds to iron-containing proteins, including prolyl hydroxylases, guanylyl cyclase, cytochrome c oxidase and globins.

In normoxic conditions, NO can inhibit PHD enzymes, thus reducing HIF-1α proteasomal degradation and promoting non-hypoxic HIF-1α signaling (Berchner-Pfannschmidt et al., 2007, Kimura et al., 2000, Metzen et al., 2003). NO can mediate cytotoxicity by reacting with superoxide, thus generating highly reactive peroxynitrite ($ONOO^-$). Accumulation of $ONOO^-$ results in nitrosative stress. Under ischemic conditions, when NOS is impaired, NO can be produced by nitrite in a non-enzymatic reaction (Zweier et al., 1999). NO is involved in many other processes, including neurotransmission (Schuman and Madison, 1991), vascular tone regulation (by stimulating NO-sensitive guanylyl cyclase) (Forstermann et al., 1986), gene transcription (Gudi et

al., 1999) and mRNA translation (e.g. by binding to iron-responsive elements) (Pantopoulos and Hentze, 1995). Therefore, its dysregulation can contribute to pathologies such as stroke, Alzheimer's disease, multiple sclerosis and atherosclerosis (Knott and Bossy-Wetzel, 2009, Naseem, 2005).

In the body, NO can be produced by a spontaneous reaction of nitrite in acid conditions, that leads to the formation of nitrous acid (HNO_2) (Eq.1). These species exist in equilibrium with oxygen intermediates (Eq.2-3), including dinitrogen trioxide (N_2O_3), which breaks down to form nitrogen dioxide (NO_2) and NO (Eq.4).

$H^+ + NO_2^- \longleftrightarrow HNO_2$ Equation 1

$HNO_2 \longleftrightarrow NOOH$ Equation 2

$NOOH + NO_2^- \longleftrightarrow N_2O_3 + OH^-$ Equation 3

$N_2O_3 \longleftrightarrow NO_2 + NO$ Equation 4

However, this process accounts for only 15-20% of the total NO produced from nitrite (Omar and Webb, 2014). Most of the NO is generated by a host of proteins that display nitrite reductase activity, including NOS enzymes, mitochondrial proteins, cytochrome P450 and heme-associated globins.

All the mammalian heme-containing globins display both nitrite reductase and NO dioxygenase activity. NO scavenging and NO formation reactions require an electron supply and depend on oxygen partial pressure (pO_2) (Figure 9). The nitrite reductase activity favors in low oxygen condition, when the globin is in the deoxy-form, whereas the NO dioxygenase activity takes place in the oxy-state. After NO dioxygenation or nitrite reduction reactions, the heme carries the oxidized Fe(III) form. The ferric heme can be reduced back to the initial ferrous form by other proteins or reducing agents, allowing for catalytic NO production or scavenging (Smagghe et al., 2008) (Figure 9).

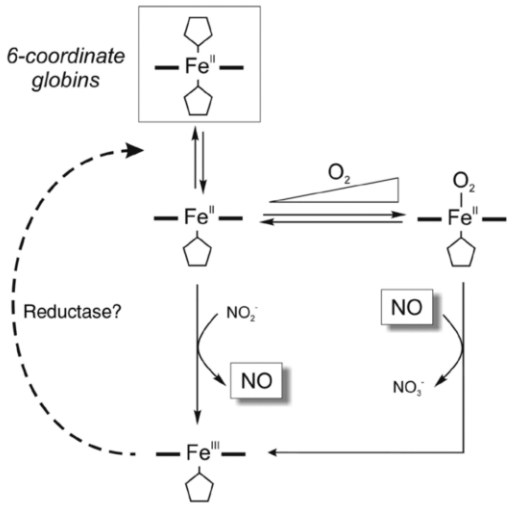

Figure 9. Schematic representation of globin-regulated NO metabolism. NO generation and NO denitrosylation pathways are regulated by environmental oxygen and globin oxygen binding affinity. The ferric heme can be reduced back to the initial ferrous form by other proteins or reducing agents, allowing for catalytic NO production or scavenging. In six coordinate globins, availability of the ferrous heme form is determined by the binding of the distal His (Tejero and Gladwin, 2014).

NO scavenging

The NO dioxygenation (Equation 5) is the most common reaction of NO with heme-containing proteins (Tejero and Gladwin, 2014).

$$Fe(II)O_2 + NO \longleftrightarrow Fe(III) + NO_3^- \quad \text{Equation 5}$$

$$Fe(II) + NO \longleftrightarrow Fe(III)NO \quad \text{Equation 6}$$

$$Fe(III) + NO \longleftrightarrow Fe(III)NO \quad \text{Equation 7}$$

$$Fe(III) + NO + OH^- \longleftrightarrow Fe(III) + NO_2^- + H^+ \quad \text{Equation 8}$$

This reaction involves the oxygenated form of the globin. At very low pO_2, the oxygen binding equilibrium will shift towards the deoxy form (FeII), which usually binds NO with high affinity (Equation 6). Due to the very slow NO release rate, this binding can decrease NO bioactivity, without any chemical modification. The last NO scavenging reaction (Equation 7) is the binding of NO to the metglobin (FeIII) form. This reaction is an equilibrium that generally favors NO dissociation from the FeIIINO complex, but particular conditions can lead to the formation of enough FeIIINO as to carry out reductive nitrosylation reactions, generating deoxy-globin and nitrite (Equation 8)

Nitrite reductase activity

The generation rate of NO from nitrite by heme-proteins depends on O_2 tension, pH, temperature and NO affinity. The nitrite reduction rate constant of different deoxy-hemeproteins is reported in Table 2 (Li et al., 2012).

Table 2. Comparison of nitrite reduction rate constants among different globins.

Hemeprotein	**Nitrite reduction rate constant (M^{-1} s^{-1})**
Cytoglobin (human)	0.14, 25°C, pH 7.0
Hemoglobin (human adult)	0.12 at T state, 6 at R state, 25°C, pH 7.4
Myoglobin (horse heart)	6, 25°C, pH 7.4
Neuroglobin (human)	0.12, 25°C, pH 7.4
Cytochrome c (horse heart)	0.07, 25°C, pH 7.4

In the deoxy-form, globins react with NO_2^-, oxidating its iron (II) to (III) and generating MetFe(III)-globin and nitrate NO_3^-. In the MetFe(III)-state, globins can form N_2O_3 by reacting with NO, or can generate MetFe(III)-NO. All these reactions are summarized in Table 3 (Lundberg and Weitzberg, 2005).

Table 3. Summary of reactions of nitrite and NO with deoxy-, oxy- and met-heme.

	NO_2^-	**NO**
DeoxyFe(II)	MetFe(III) + NO	Fe(II)–NO (iron-nitrosylated Hb)
OxyFe(II)	MetFe(III) + NO_3^-	MetFe(III) + NO_3^-
MetFe(III)	MetFe(III)–NO_2^- (reversible) (+ NO → N_2O_3)	MetFe(III)–NO (reversible)

Hexa-coordinated globins (i.e. Cygb, Ngb) require hexa-to-penta-transformation to allow the binding of NO_2^- to the heme and perform nitrite reductase activity (Omar and Webb, 2014). The mechanism of nitrite reduction by Cygb (and Ngb) is similar to that of Hb and Mb and requires a three-step reaction (Li et al., 2012):

$$H^+ + NO_2^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HONO$$

$$Fe^{2+} - Cygb + HONO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fe^{3+} - Cygb + NO + OH^-$$

$$Fe^{2+} - Cygb + NO \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} NO - Fe^{2+} - Cygb$$

However, the nitrite reductase activity of Cygb is much weaker than Hb and Mb, because only 0.6% of Cygb displays heme penta-coordination to react with NO_2^- (Li et al., 2012) Additionally, Cygb concentrations in cells, such as smooth muscle, are in the micromolar range (~1–10 µM), too low to guarantee a significant NO production. However, Cygb upregulation upon hypoxia (up to an order of magnitude (Avivi et al., 2010, Schmidt et al., 2004)) increases its contribution to NO production to >60% (Li et al., 2012). Consistent with its potential involvement in NO metabolism, Cygb has been shown to co-localize with nNOS in certain nerve populations in the mouse brain (Hundahl et al., 2010).

1.6.5.4 Anti-oxidative function of Cygb

ROS include radical and non-radical oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO·). Cellular ROS are generated endogenously by mitochondria, during the process of oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds. Oxidative stress occurs when ROS overwhelm the cellular antioxidant defense system, either through an increase in ROS levels or a decrease in the cellular antioxidant capacity. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and has been implicated in different diseases, including carcinogenesis (Szatrowski and Nathan, 1991, Toyokuni et al., 1995), neurodegeneration (Butterfield and Lauderback, 2002), atherosclerosis, diabetes, and aging (Harman, 1956). However, ROS involvement in the pathogenesis of disease states is not confined to macromolecular damage. There is increasing evidence that ROS are important cellular mediators involved in regulating signaling pathways (Lo et al., 1996, Rhee et al., 2003).
Cygb expression is upregulated in response to oxidative stress, i.e. upon H_2O_2 treatment in breast cancer and neuroblastoma cell lines (Chua et al., 2009, Li et al., 2007). Additionally, overexpression of Cygb may protect cells subjected to oxidative stress, promoting cell viability (Fordel et al., 2007b, Hodges et al., 2008, Xu et al., 2006), ROS and lipid-derived radicals detoxification (Fordel et al., 2007b, Hodges et al., 2008, Mimura et al., 2010, Xu et al., 2006) and reducing DNA damage (Fordel et al., 2007b, Hodges et al., 2008, Mimura et al., 2010, Trandafir et al., 2007, Xu et al., 2006). On the other hand, Cygb depleted cells showed an increased apoptotic rate and ROS accumulation (Fordel et al., 2007a). The molecular mechanism underlying the anti-oxidative function is still unclear. Cygb may scavenge ROS using heme and thiol residues (Fordel et al., 2007a, Petersen et al., 2008). Hypothesized physiological functions of Cygb included peroxidase and superoxide dismutase activity, which were subsequently excluded due to the lower tissue concentration of Cygb compared to known enzymes with similar role.

Cygb can bind lipids and promote their peroxidation *in vitro* (Reeder et al., 2011). It has been hypothesized that lipids or lipid-like molecules could affect the globin–heme coordination state, leading to its transition from the hexa- to penta-coordinated heme-iron state. This conformational change could be regulated by the redox state of the Cys38 and Cys83 residues (Beckerson et al., 2015, Reeder et al., 2011). Recently, Tejero and colleagues demonstrated that anionic phospholipids, particularly phosphatidylinositol phosphates, can affect structural organization of Cygb and modulate its iron state and peroxidase activity both in presence or absence of cysteine oxidation (Tejero et al., 2016). The mechanism, *in vivo* validation and biological relevance of Cygb-mediated lipid peroxidation remains to be further investigated.

1.6.5.5 Collagen synthesis

Collagen is the main structural component of connective tissue and the most abundant protein in mammals. Extensive collagen deposition is one of the main characteristic of organ fibrosis. Cygb was first identified in hepatic stellate cells of fibrotic liver, and its expression was induced by fibrosis and correlated with the accumulation of collagen I (Kawada et al., 2001). Upon fibrogenic stimulus, an increased Cygb expression and collagen synthesis was also observed in pancreatic stellate cells and renal interstitial cells (Mimura et al., 2010, Zion et al., 2009). Moreover, overexpression of the Cygb in combination with TGF-β enhanced collagen production in rat fibroblasts *in vitro* (Nakatani et al., 2004). Cygb is detected in fibroblasts, chondroblasts and osteoblasts, which actively secrete extracellular matrix components (Schmidt et al., 2004). In addition, while hypoxia upregulates Cygb expression, it also triggers organ fibrosis, wound healing, osteogenesis and chondrogenesis, which require massive, O_2-dependent collagen production.

It has been hypothesized that Cygb might supply O_2 to collagen prolyl hydroxylase, a key enzyme in the biosynthesis of collagen (Hankeln et al., 2005). However, due to the lower O_2 affinity of prolyl-hydroxylases (Hirsila et al., 2003) compared to Cygb, the putative Cygb role as O_2 donor has been excluded. Cygb overexpression can reduce collagen deposition in fibrotic liver (Xu et al., 2006) and kidney (Mimura et al., 2010), suggesting a putative anti-fribrotic role of Cygb. *In vitro* data support these findings in kidney cell lines (Mimura et al., 2010), lung and breast cancer cell lines (Shivapurkar et al., 2008). On the other hand, Cygb knock down did not affect collagen expression in kidney cell lines (Mimura et al., 2010).

The association between Cygb and collagen expression is complex and might dependent on cell type, genetics and molecular environment. The mechanisms involved in this association is still unknown. An interplay between Cygb, NO and ascorbate has been suggested. Ascorbate serves

as a cofactor for collagen hydroxylation, whereas NO inhibits collagen production e.g., via inhibition of prolyl hydroxylase activity and upregulation of matrix metalloproteinases (Cao et al., 1997, Dooley et al., 2007). However, NO can also induce collagen synthesis e.g. in wound healing (Murrell, 2007, Witte and Barbul, 2002). The association between Cygb and collagen production requires further investigation, as it implies that the globin may be involved in tissue regeneration processes and, thus, may be useful as a potential pharmacological target in the future.

1.7 Disorders associated with Cygb

Increasing evidence indicate Cygb involvement in the pathogenesis of different diseases. Cygb seems to be important in the maintenance of normal phenotype, protecting cells from stress conditions. Endogenous upregulation of Cygb occurs in glaucoma areas (Ostojic et al., 2006), fibrotic liver (Kawada et al., 2001), gastroesophageal reflux disease (McRonald et al., 2006), in neurons of patients with ferritinopathy (Powers, 2006), in cytoplasmic inclusion in the neocortex of patients with psicomotor retardations and/or epilepsy (Hedley-Whyte et al., 2009). On the other hand, a reduction in Cygb expression levels has been observed in different cancer types, including esophageal, lung, head, neck (McRonald et al., 2006, Shaw et al., 2006, Shaw et al., 2009, Shivapurkar et al., 2008, Xinarianos et al., 2006). Interestingly, a common link of all these disorders is their association with hypoxic, nitrosative and oxidative stress. So far, no disease-causing mutations in the *CYGB* gene have been identified in GWAS.

1.7.1 Cygb in neurodegenerative disorders

Cygb upregulation in neurodegenerative disorders might be linked to stress-related conditions. Ischemia and nitrosative stress lead to nerve injury and glaucoma development (Mozaffarieh et al., 2008). Immunohistochemistry of brain tissue from patients with epilepsy and/or psychomotor retardation showed positive Cygb staining within astrocytic inclusions. Although the relationship between inclusions and seizures is not confirmed, it cannot be excluded. Redox alteration in nerves can derive also by mitochondrial dysfunction and might contribute to a seizure-prone phenotype. One can speculate that Cygb upregulation might be induced by oxidative stress, possibly via NF-κB signaling. Alternatively, Cygb could be kept in astrocytic inclusion thus preventing its function in redox homeostasis and leading to an increase in redox unbalance and chronic oxidative/nitrosative stress.

1.7.2 Cygb in fibrosis

Cygb was upregulated in rat hepatic stellate cells (HSCs) upon fibrosis stimulation (Kawada et al., 2001). In normal conditions, HSCs are involved in blood flow control and vitamin A storage. In injured liver, activated HSCs transform into myofibroblasts and undergo deposition of extracellular matrix components, leading to organ fibrosis (Tateaki et al., 2004). Higher level of Cygb have been found in other models of fibrotic liver (Bosselut et al., 2010, Gnainsky et al., 2007, Man et al., 2008, Tateaki et al., 2004), kidney (Mimura et al., 2010) and pancreas (Nakatani et al., 2004, Zion et al., 2009). Notably, Cygb induction is frequently concomitant with increase in collagen expression (Nakatani et al., 2004). Additionally, exogenous overexpression of Cygb prevent liver fibrosis, reducing HSCs activation, liver necrosis and collagen depositions (Xu et al., 2006). Similar anti-fibrotic and protective roles were observed in Cygb transgenic rats subjected to subtotal nephrectomy (Mimura et al., 2010). Cygb overexpression reduced loss of renal function, kidney fibrosis, collagen deposition macrophage infiltration, fibroblast activation and oxidative/nitrosative stress (Mimura et al., 2010).

The underlying mechanism of cytoprotection is still unknown. Cygb might have an antioxidant role, since Cygb overexpression has been associated to fibrosis reduction and treatment with antioxidants has anti-fibrotic effect. However, the ROS scavenging function of Cygb associated to its anti-fibrotic role needs further investigations.

1.7.3 Cygb in cancer

Cygb association to cancer is controversial. Several studies have associated Cygb downregulation with tumor progression, whereas other malignances display Cygb overexpression. This bimodal tumor suppression and oncogene behavior, possibly cell-type-specific, is still not clear. In lung cancer cell lines, Cygb revealed tumor suppressor properties in normoxia, but promoted tumorigenic potential of the cells exposed to stress, suggesting a bimodal function in lung tumorigenesis, depending on cell type and microenvironmental conditions (Oleksiewicz et al., 2013).

Cygb tumor suppressor activity was demonstrated in lung, esophageal, head, neck, breast, bladder and colon. Mechanism of Cygb downregulation in esophageal (McRonald et al., 2006), ovarian (Presneau et al., 2005) and non-small cell lung cancer (NSCLC) (Xinarianos et al., 2006) include promoter hypermethylation and loss of heterozygosity (LOH) at chromosome 17q25 (McRonald et al., 2006). In particular, *CYGB* LOH was found more frequently in poorly differentiated tumors, while *CYGB* promoter methylation occurs preferentially in adenocarcinomas (Xinarianos et al., 2006).

CYGB promoter hypermethylation and the corresponding mRNA downregulation have been observed also in independent studies on head and neck squamous cell carcinoma (Shaw et al., 2006, Shaw et al., 2009, Xinarianos et al., 2006), as well as in oral epithelia dysplasia (Hall et al., 2008), leukemia, breast colon and bladder cancers (Shivapurkar et al., 2008). The high frequency of Cygb promoter hypermethylation across various tumor types implies that this is an early event during carcinogenesis (Hall et al., 2008, Shivapurkar et al., 2008).

The molecular mechanism of tumor suppressor gene of Cygb is still unknown. It might be speculated that Cygb acts as antioxidant, reducing the cancer-mediated oxidative and nitrosative stress, thus protecting the cells from injuries at DNA, protein and membrane level. In the esophageal tumor, loss of Cygb could lead to impaired tissue regeneration that activates fibrosis and the inflammatory response. Inflammation results in increase NO synthesis, which affects p53 and mitogen-activated protein kinase pathways, promoting angiogenesis, migration, invasion and DNA damage, supporting carcinogenesis.

Despite the large amount of data supporting a tumor suppressing function of Cygb, several studies have shown that Cygb might be also up-regulated in cancers. Cygb mRNA was found to be upregulated in a subset of NSCLC (Xinarianos et al., 2006) and immunohistochemical analysis of alveolar soft-part sarcoma showed Cygb overexpression in lung and brain metastasis (Genin et al., 2008). Gorr and colleagues demonstrated that Cygb was generally lost in breast cancer compared to normal samples, although no expression changes were observed in other tumor types tested. In agreement with other studies, Cygb expression correlated with hypoxia markers such as HIF-1α, HIF-2α, CAIX and fatty acid synthase, but not with Glut1 (Gorr et al., 2011). Cygb upregulation in malignancies could be related to oxygen deficiency and activation of the hypoxia response pathway (Shaw et al., 2009).

Cancer cells frequently modulate signaling pathways to promote cell survival, angiogenesis and resistance to chemo and radio therapy. Cygb can modulate NO by its NO dioxygenase function affecting HIF-1α stability and signaling (Berchner-Pfannschmidt et al., 2008, Berchner-Pfannschmidt et al., 2007, Huang et al., 1999, Metzen et al., 2003). Cygb could take part in the adaptive process that leads to a more aggressive phenotype, by protecting cancer cells against hypoxia, oxidative and/or nitrosative stress. If this hypothesis is correct, Cygb, like few other genes (i.e. TGF-β) could behave as both tumor suppressor and oncogene. A recent publication by Kawada's group, using Cygb knock out mice, reported on the development of several age-dependent abnormalities including tumors, in accordance with a tumor suppressor function of Cygb (Thuy le et al., 2016). Further investigations are required to elucidate the mechanism by which Cygb contributes to the molecular pathogenesis of human disease, including cancers.

1.8 Cygb mouse models

Only few studies published so far involve the use of Cygb mouse models. A skeletal muscle-specific Cygb knockout mouse line (smCygb$^{-/-}$) was generated in Mammen's lab (Singh et al., 2014), using the standard Cre/LoxP technology, i.e. crossing homozygous floxed mice (Cygb$^{flox/flox}$) with Myo-Cre transgenic mice. In this study, Singh and colleagues investigated the role of CYGB in muscle regeneration by comparing the ability of WT and smCygb$^{-/-}$ mice to recover upon muscle injury and oxidative stress. They demonstrated that Cygb has a protective role in myogenic progenitor cell viability and differentiation and suggest it is therefore important for myogenesis and skeletal muscle regeneration (Singh et al., 2014).

Cygb global knock out (Cygb$^{-/-}$) mice were generated in the lab of Kawada by deleting exon 1 of the mouse *Cygb* gene and backcrossed on the C57BL/6J background (Thuy le et al., 2011). Cygb$^{-/-}$ mice appeared normal at morphological and histopathological level 1 month after birth (Thuy le et al., 2011), as observed for other globins like Mb (Garry et al., 1998) and Ngb (Hundahl et al., 2011). Our knowledge about Cygb$^{-/-}$ mice phenotype derives from Kawada and his group that focused their studies on the liver. They reported that Cygb$^{-/-}$ mice subjected to *N,N*-diethylnitrosamine (DEN) treatment were highly susceptible to tumor development in liver and lungs (Thuy le et al., 2011). Furthermore, Cygb$^{-/-}$ mice displayed increased liver fibrosis, inflammation and development of hepatocarcinoma in a non-alcoholic steatohepatitis model induced by a choline-deficient L-amino acid-defined diet via activation of the oxidative stress pathway (Thuy le et al., 2015).

Very recently, Kawada's group reported on the short- and long-term effects of Cygb deficiency, comparing young and aged mice. Under 1 year of age (young) Cygb$^{-/-}$ mice showed heart hypertrophy, cysts in kidney and ovary and less frequently loss of balance, paralysis, liver fibrosis and lymphoma (Thuy le et al., 2016). Cygb$^{-/-}$ mice from 1-2 years of age (aged) displayed multiple organ abnormalities, including heart hypertrophy and tumors in liver, lung, small intestine, ovary and lymphatic organs (Thuy le et al., 2016). Cygb deficiency was associated with increasing oxidative and nitrosative stress, fibrosis, inflammation, DNA damage and cellular senescence. Additionally, the absence of Cygb increases NO metabolites in the urine and serum of Cygb$^{-/-}$ mice, suggesting a role in NO metabolism (Thuy le et al., 2016).

1.9 Other globins

1.9.1 Globin E

Globin E (GbE) is an eye-specific globin first described in chicken in 2004 (Kugelstadt et al., 2004) and later found in other bird genomes (Blank et al., 2011a, Hoffmann et al., 2011, Storz et al., 2011). Initially GbE was thought to be a bird-specific globin, but further investigations identified GbE orthologues in the prehistoric fish coelacanth (Schwarze and Burmester, 2013) and in turtles (Schwarze et al., 2015). Phylogenetic analysis showed that GbE is an ancient globin, related to Mb (Blank et al., 2011a, Hoffmann et al., 2011) that is able to bind O_2. Immunohistochemistry studies indicated that GbE is mainly expressed in the chicken's eye with a concentration of ~10 μM in total eye tissue, specifically in the outer segments of photoreceptors (Blank et al., 2011a). Putative functions of GbE include O_2 supply to the retina (Blank et al., 2011a).

1.9.2 Globin X

Globin X (GbX) is a membrane-associated globin expressed in metazoans and in lower vertebrates such as lampreys, sharks, coelacanth, reptiles, amphibians, but not in mammals and birds (Blank and Burmester, 2012, Fuchs et al., 2006, Roesner et al., 2005). A GbX-like paralogue is expressed only in invertebrates (Blank and Burmester, 2012, Hoffmann et al., 2012). GbX primary sequences are longer than other globins and count ~200 amino acids, showing extension at both N- and C- terminals. The core sequence of GbX is highly conserved whereas the extension sequences are variable. GbX is a hexa-coordinated globin that binds to membranes due to N-terminal acylation sites (palmitoylation at Cys3 and myristoylation at Gly2) (Blank et al., 2011b). The function of GbX is still unknown, but putative roles include protection of membrane lipids, or involvement in membrane-associated signaling pathways (Blank et al., 2011b). In zebrafish, GbX localizes in the CNS and could have a role in the sensory process (Blank et al., 2011b). Recently, GbX have been found in erythrocytes of bass and zebrafish, in addition to Hb (Corti et al., 2016). Deoxy-GbX displays fast nitrite reduction rate (200-fold higher than human Hb) and it reduces nitrite to form NO thus inhibiting platelet activation *in vitro* (Corti et al., 2016). The physiological relevance of these findings remains to be determined as expression levels of GbX are much lower compared to Hb.

1.9.3 Globin Y

GbY was originally identified in *Xenopus* (Fuchs et al., 2006) and subsequently found in other species like platypus, lizards, turtles, coelacanth, but not in higher mammals (Marsupialia and

Placentalia) or birds. The expression profile of GbY changes during development, showing peaks during organogenesis and metamorphosis (Schwarze et al., 2015). In the adult *Xenopus* GbY is expressed in kidney, eye, lung and ovary (Fuchs et al., 2006). Its function remains unknown due to the lack of functional studies.

1.9.4 Androglobin

Adgb represents the last member added to the globin superfamily and was discovered in 2012 (Hoogewijs et al., 2012b). Adgb belongs to a family of chimeric proteins with an N-terminal protease domain, a central globin-like domain and a IQ calmodulin binding motif. The name “Adgb” was chosen because of its preferential localization in testes.

The sequence of Adgb is exceptionally long, comprising ~1600 amino acids and is highly conserved in different species including mammals, vertebrates, all metazoan and choanoflagellates, suggesting an early evolutionary origin. Interestingly, Adgb is not present in the model organisms *D. melanogaster* and *C. elegans*. The latter is surprising given the presence of more than 30 globins in *Caenorhabditis* (Hoogewijs et al., 2008, Hoogewijs et al., 2007). Adgb is expressed most abundantly in testes, particularly during the postmeiotic stages of spermatogenesis, but also in lungs to a smaller extent. Preliminary data from a newly generated Adgb knock-out mouse model showed that lack of Adgb is associated with male infertility, due to absence of mature spermatozoa, suggesting a key role of Adgb in reproduction. Additionally, analysis of Adgb levels in testes and semen from fertile and infertile men illustrated a correlation between low Adgb expression and infertility (Santambrogio et al., unpublished data).

1.10 Diabetes and kidney complications

Diabetes mellitus or simply diabetes is one of the most common chronic diseases worldwide and continues to increase in incidence. As per 2014 estimates, the global prevalence of diabetes was 8.3%, affecting more than 387 million adults worldwide. The number of patients are expected to rise to as much as 55% by the end of the year 2030, with almost 600 million adults affected (Whiting et al., 2011). Diabetes comprises a group of metabolic disorders characterized by persistent hyperglycemia due to chronic and/or relative insulin insufficiency (Mathis et al., 2001). Insulin is a peptide hormone produced by the β-cells of the Langerhans islets in the pancreas and plays a role in maintaining constant glucose levels in the blood. The destruction of β-pancreatic cells and therefore insufficient insulin production and/or insulin resistance from the tissues lead to hyperglycemia (Alberti and Zimmet, 1998). There are two major forms of diabetes, named type I

(insulin-dependent) and type II (insulin-independent), although diabetes may occur during pregnancy or other conditions, including drug toxicity, insulin receptor disorders and genetic mutations (Association, 1997). In type I diabetes, genetic and environmental factors lead to an autoimmune response responsible for pancreatic β-cell destruction and therefore insulin-deficient hyperglycemia (Atkinson and Maclaren, 1994). While type I diabetes occurs in children and young adults, type II arises in all ages and covers 85% of diabetic cases. Type II diabetes develops when pancreatic β-cells fail to secrete sufficient amounts of insulin to meet the metabolic demand. The major cause of insulin deficit is the development of resistance by several tissues including liver, skeletal muscle and adipose tissue. In order to maintain normal glucose levels, pancreatic β-cells increase their insulin secretion, but only temporary. This compensatory hypersecretion is then followed by β-cells failure, insufficient insulin production and therefore hyperglycemia (Kasuga, 2006). Type II diabetes is a complex disease in which both genetic and environmental factors play important roles. “Western life style” and high caloric diet increase the global incidence of type II diabetes as well as obesity in developing countries. In fact, 60 to 90% of type II diabetes cases are associated with obesity (Colditz et al., 1990, Wolf and Colditz, 1998). Poor glycemic control associated to diabetes increase the risk of both acute and chronic complications. Acute metabolic disorders includes diabetic ketoacidosis as a result of hyperglycemia and coma due to hypoglycemia (Murphy, 1998). Long-term complications of diabetes comprise diabetic nephropathy, neuropathy, retinopathy and cardiovascular complications, such as heart attack and stroke (Bailes, 2002). The latter occur as consequence of kidney disease, in addition to loss of erythropoietin, vitamin D deficiency, dysregulation of blood pressure and fluid balance (Taal, 2012).

1.10.1 Kidneys in health and diabetic nephropathy

The kidneys play a major key role in in regulating many important body functions, including maintaining an appropriate body volume, regulating the ionic composition of the blood and its acid-base homeostasis (blood pH). Kidneys regulate blood pressure homeostasis and red blood cell formation by producing hormones such as renin and erythropoietin, respectively. Additionally, kidneys are responsible for the excretion of metabolic waste products and chemicals in the urine. Each human kidney counts ~1 million nephrons, the functional unit of the kidney. Nephrons are located between the kidney cortex and medulla and consist of a glomerulus and a tubular system. The glomerulus comprises a capillary tuft located inside the Bowman’s capsule (Figure 10B) and is responsible for plasma filtration. Subsequently, a tubular system that spans from the cortex to the kidney medulla (Figure 10A) is responsible for the reabsorption of useful substances. After

passing through the renal tubule, the filtrate reaches the collecting duct for urine elimination (Figure 10B).

Every day, ~180 liters of primary urine is normally formed by the kidney glomeruli. The tubular system allows the reabsorption of water and electrolytes, such that the final daily excretion is normally only ~1-1.5 liters.

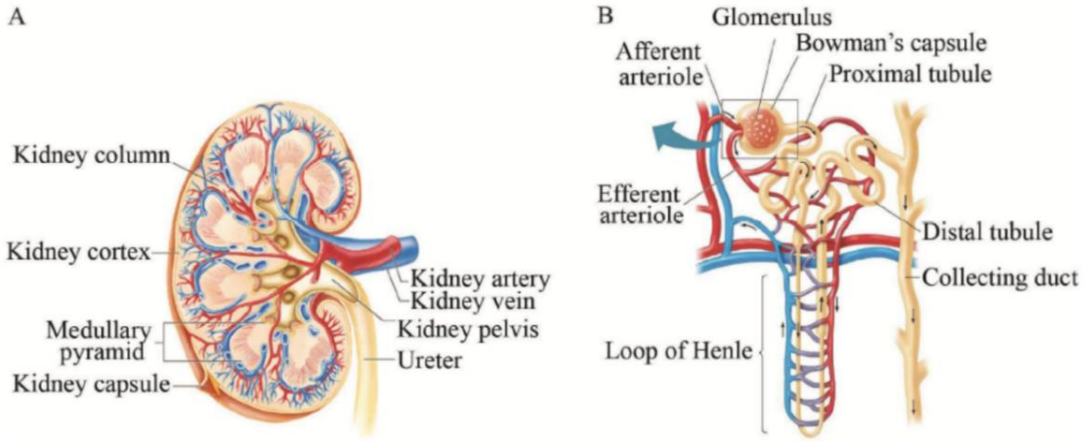

Figure 10. Schematic illustration of A. kidney and B. nephron anatomy. Adapted from (Marieb, 2001).

Glomerular filtration barrier

In the 1960s-1970s, tracer molecules of different sizes and charges were used to identify the nature of the glomerular filtration barrier (GFB) (Kanwar and Farquhar, 1979, Kanwar et al., 1980, Rennke et al., 1975). The size- and charge-selective ultrafiltration of plasma occurs in glomeruli (Figure 11). Primary urine is filtrated from the plasma through the GFB to the Bowman's space and then passes to the tubular system. While water and small molecules pass the barrier, albumin (~67 kDa) and protein with similar or bigger size are retained in the blood. The GFB consists of three distinct layers: the fenestrated endothelium, the glomerular basement membrane (GBM) and the podocytes with their slit diaphragms (SD) (Figure 11).

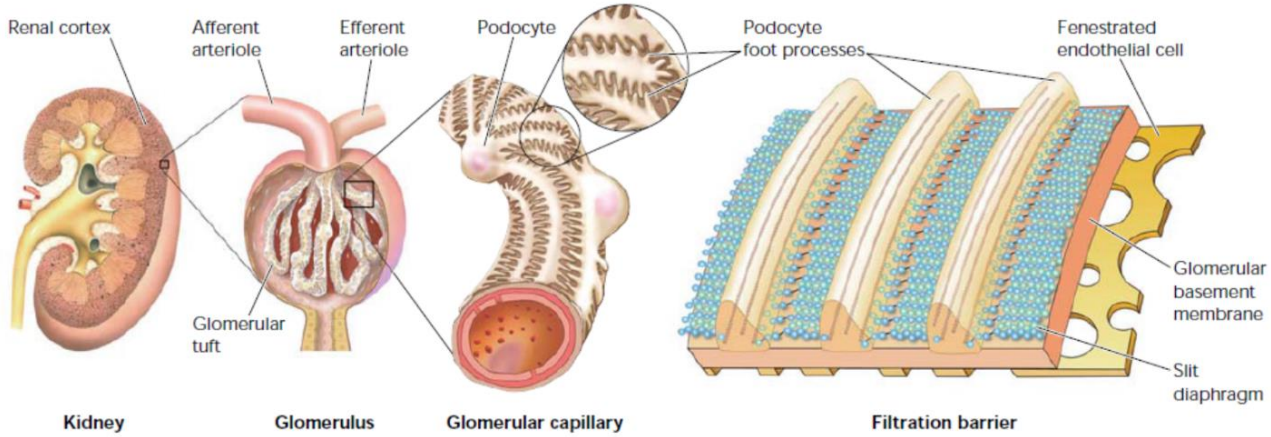

Figure 11. Renal glomerular filtration system. An afferent arteriole branches into capillaries (glomerular tuft). The filtration takes place across the capillaries wall: the plasma filtrate (primary urine) is led to the proximal tubules while unfiltered blood is returned to the blood circulation, via an efferent arteriole. The filtration barrier contains fenestrated endothelial cells, the glomerular basement membrane and podocytes with their interdigitating foot processes forming the slit diaphragm (Tryggvason and Wartiovaara, 2005).

Endothelium

The endothelium of glomerular capillaries displays numerous fenestrations of 70–100 nm in diameter and provides the first structural barrier to albumin. Negatively charged proteins such as proteoglycans, glycosaminoglycans, glycoproteins and glycolipids form the glycocalyx, that contributes to the charge-selective properties of the GFB (Avasthi and Koshy, 1988). The endothelium is involved also in the formation of the GBM by synthesizing laminin (St John and Abrahamson, 2001) and type IV collagen (Abrahamson et al., 2009). The endothelial cells communicate with the podocytes and the mesangial cells through a variety of signaling pathways to prevent proteinuria (Daehn et al., 2014, Davis et al., 2007).

Glomerular basement membrane

The GBM is an amorphous, 300- to 350-nm-thick extracellular structure that is surrounded and maintained by endothelial cells and podocytes. The GBM can be separated into three layers: lamina rara externa (outer, underneath the foot processes), lamina densa (central) and lamina rara interna (inner, subendothelial). The main components of the GBM include type IV collagen, laminin, nidogen (entactin) and the negatively charged heparan sulfate proteoglycans (HSPGs) which contribute to the charge-selectivity of the GFB (Avasthi and Koshy, 1988, Groffen et al., 1998).

Mesangium

The mesangium consists of mesangial cells and their surrounding matrix and is located between the capillary loops of the glomerular tuft. The major role of the mesangium is to provide structural support for the glomerular capillaries. Mesangial cells resemble smooth muscle cells by having contractile properties and they participate in the regulation of glomerular filtration (Becker, 1972) (Ausiello et al., 1980). They also control the composition and turnover of the mesangial matrix and contribute to the removal of glomerular debris due to their phagocytic activity (Elema et al., 1976, Mauer et al., 1972).

Podocytes

Podocytes are polarized, highly specialized and terminally differentiated cells that surround the glomerular capillaries and form a key part of the GFB.

With respect to their cytoarchitecture, podocytes consist of three different segments. The cell body gives rise to major processes that branch into a network of interdigitating cellular extensions called foot processes. The foot processes of neighboring podocytes interdigitate between the basal and apical sides of the podocytes with specialized 40-nm-wide cell-to-cell junctions called slit diaphragms (SD) (Furukawa et al., 1991). The SD is highly permeable to water and small molecules, but not to larger proteins, like albumin, and represents the main size-selective filter of the GFB (Boute et al., 2000, Donoviel et al., 2001, Karnovsky and Ainsworth, 1972). Podocyte injury leads to foot processes effacement. Thus, during proteinuric diseases like diabetic nephropathy, the foot processes lose their fine structure and collapse.

Slit diaphragm

The SD represents the only cell-cell contact between podocytes. The major component of the SD is nephrin (Ruotsalainen et al., 1999). Nephrin is a transmembrane protein of the immunoglobulin (Ig) superfamily and, together with the Neph-protein family (Neph 1-3), forms a framework of the SD (Barletta et al., 2003, Gerke et al., 2003, Gerke et al., 2005, Ruotsalainen et al., 1999) (Wartiovaara et al., 2004). Based on its components, the SD is considered as a special cell adhesion structure with features of both adherens and tight junctions (Reiser et al., 2000). Adherens junctions mediate cell to cell contact. The adherens junction proteins present in SD include cadherin family proteins placental (P)-cadherin (Reiser et al., 2000) and vascular endothelial (VE)-cadherin (Cohen et al., 2006), as well as catenins (Reiser et al., 2000) and cadherin-like protein FAT1 (Inoue et al., 2001). The tight junction associated proteins detected in the SD include Zonula occludens-1 (ZO-1) (Schnabel et al., 1990), membrane associated guanylate kinase inverted-1 (MAGI-1) (Hirabayashi et al., 2005), MAGI-2 (Lehtonen et al., 2005), calmodulin associated serin/threonine kinase (CASK) (Lehtonen et al., 2004) and junction adhesion molecule-1 (JAM-1) (Fukasawa et al., 2009). In addition, several other molecules, including cytoplasmic CD2 adapter protein (CD2AP) (Shih et al., 1999) and podocin (Schwarz et al., 2001), have been shown to be connected to the SD.

The slit diaphragm sends signals regulating cell polarity, cell survival and cytoskeleton organization. Phosphorylation of nephrin and Neph1 initiates signaling pathways in podocytes, and podocin contributes to intracellular signal transduction via interaction with nephrin (Boute et al., 2000, Huber et al., 2003, Li et al., 2004). CD2AP, together with nephrin, activates PI3K and

stimulates serine/threonine kinase AKT-dependent signaling (Huber et al., 2003, Li et al., 2000). CD2AP and ZO-1 also appear to connect the SD to the actin cytoskeleton (Fanning et al., 2002, Lehtonen et al., 2002) (Huber et al., 2003).
Mutations in each of these proteins have been associated to nephrotic syndrome-like phenotype, i.e. proteinuria (Boute et al., 2000, Donoviel et al., 2001, Kestila et al., 1998), indicating their crucial role in maintaining the SD structure.

1.11 Diabetic nephropathy

Diabetic nephropathy (DN) is one the most prevalent chronic complications of diabetes and represents the leading cause of chronic kidney disease (CKD). DN is responsible for 30-40% of all end-stage renal disease (ESRD) cases in industrialized countries, e.g. 44% in the US(Collins et al., 2015). The majority of diabetic patients subjected to renal replacement therapy (e.g. dialysis, hemofiltration and ultimately renal transplantation) displays type II diabetes, as its incidence is much higher compared to type I (Collins et al., 2015).

1.11.1 Pathogenesis of DN

DN is characterized by persistent albuminuria (>300 mg of albumin per 24 hours or 300 µg/g creatinine) and progressive decline in glomerular filtration rate (GFR). Early symptoms of DN include peripheral edema caused by capillary hypertension (Hommel et al., 1990) and anemia (Ritz and Haxsen, 2005). Hyperglycemia is responsible for both microscopic and ultramicroscopic changes in the kidney structure, which involve glomeruli, tubuli, interstitium and vessels.
The main ultrastructural and earliest detectable change in diabetic kidneys is the thickening of the GBM due to increased accumulation of extracellular matrix (ECM), as a consequence of an increased production or reduced degradation (Schnaper et al., 1996). Additionally, interstitial and mesangial accumulation of ECM components, such as types IV and VI collagen, laminin and fibronectin occurs. Mesangial expansion associates with reduced filtration surface area in glomeruli and, consequently, with reduced GFR and progressing albumin excretion (Fioretto et al., 1995, Kimmelstiel and Wilson, 1936, Mauer et al., 1984). Ultramicroscopic changes comprise effacement of podocytes and decrease in their density, due to apoptosis and/or detachment (Susztak et al., 2006) (Figure 12).
Additional features of DN are tubular hypertrophy, apoptosis, thickening of the tubular basement membrane (Brito et al., 1998) and, proportional to tubular atrophy, interstitial fibrosis (Mauer et al., 1984). Hyaline arteriolosclerosis (thickening of the wall) of afferent and efferent arterioles is also

a typical lesion in DN. However, hyalinosis of efferent arterioles is specific for DN and distinct from other kidney diseases (Stout et al., 1994).

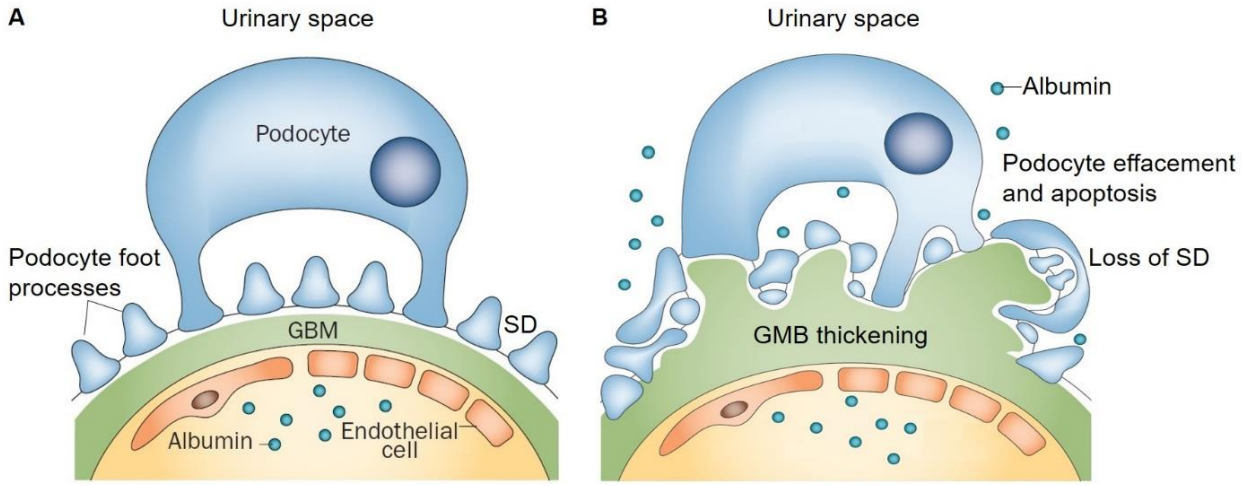

Figure 12. Glomerular filtration barrier changes during diabetic nephropathy. A) Normal glomerular filtration barrier. B) Diabetic kidney. GBM: glomerular basement membrane; SD: slit diaphragm. Modified from Suh et al.2013 (Suh and Miner, 2013).

Role of podocytes in the pathogenesis of DN

Podocyte depletion represents one of the earliest cellular lesions affecting the diabetic kidney. Podocytopathy in DN is characterized by foot process widening. Diabetic patients (Dalla Vestra et al., 2003) (Pagtalunan et al., 1997, Steffes et al., 2001, White et al., 2002) and animal models of DN (Pichaiwong et al., 2013, Susztak et al., 2006) show a decreased number of podocytes. Due to the central role of podocyte's slit diaphragm in the glomerular filtration barrier, their loss was clearly associated to elevated proteinuria (Berg et al., 1998, White et al., 2004).

As podocytes are terminally differentiated cells, analogous to the neuron, they do not proliferate *in vivo*. Their quiescent state is guarantee by a tight regulatory control of cell cycle, with a strong and specific upregulation of cyclin-dependent kinase (CDK)-inhibitors. Therefore, loss of podocytes in diabetes can be due to apoptosis and/or cell detachment. The later scenario does not exclude a role of apoptosis, since cells may first detach and then undergo apoptosis or vice versa (*anoikis* = apoptosis caused by loss of attachment of cells to their basement membrane). Alternatively, detached cells can be found in the urine as viable podocytes in patients (Vogelmann et al., 2003) and in DN-induced rats (Petermann et al., 2004).

Podocytes are connected on the basolateral membrane to the GBM via integrins (α and β subunits) (Adler, 1992) and dystroglycans (Hemler, 1999). Decreased levels of $\alpha 3\beta 1$ integrins

have been found in patients with DN and streptozotocin-induced diabetic rats (Chen et al., 2000), and altered expression of dystroglycan 1 has been observed in early DN of db/db mice, a type II diabetes model (Makino et al., 2006).

High glucose, TGF-β and angiotensin II (AngII) induce apoptosis of podocytes (Ding et al., 2002, Liu et al., 2013, Schiffer et al., 2001). AngII appears to induce apoptosis in cultured rat glomerular epithelial cells at least partially via TGF-β because its apoptotic effect is attenuated by an anti-TGF-β antibody (Steffes et al., 2001). Moreover, ROS may contribute to podocyte apoptosis and depletion upon high glucose treatment and in experimental DN (Liu et al., 2013, Susztak et al., 2006).

1.11.2 Triggers of diabetic nephropathy

The onset and progression of DN depends on multiple risk factors, including gender, age, poor glycemic control, life style (i.e. smoking, obesity), dyslipidemia, but also genetic susceptibility. However, hyperglycemia and glomerular hypertension represent the two major triggers of diabetic kidney disease.

In DN, hyperglycemia is the driving force for the progressive destruction of the glomeruli via several mechanisms. The chronically elevated levels of blood glucose lead to the formation of advanced glycation end-products (AGEs) by irreversible non-enzymatic glycation of proteins and lipids, causing glomerular hypertrophy and increased ROS.

Hyperglycemia activates protein kinase C (PKC) that results in stimulation of growth factors and decrease in endothelial NO availability, by inhibition of endothelial NOS (Harrison et al., 1996).

Increased activity of polyol pathways leads to conversion of glucose to sorbitol by aldose reductase and further, reduced NADPH oxidase. The polyol pathway plays an important role in DN pathogenesis, leading to increased oxidative stress and activation of PKC (Ramana, 2011). NADPH is also a co-factor of the enzyme glutathione reductase which maintains the cellular redox state via conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Alter et al., 2012). During sorbitol metabolism, important glycation agents, fructose-3-phosphate and 3-deoxyglucosone are also produced. Increased uptake of glucose due to polyol pathway increases AGEs production and binding of AGEs with its receptor produces ROS (Yamagishi et al., 2012). Overproduction of ROS reduces the activities of endogenous anti-oxidant enzymes, such as superoxide dismutase (SOD) and catalase, leading to oxidative stress, DNA damage, and finally cell death.

Hyperglycemia also produces mechanical tension and frictional force which occur in tandem as a result of the hemodynamic changes to the glomeruli, which leads to the liberation of numerous

cytokines, pro-inflammatory markers and growth factors which stimulate various pathways of oxidative stress (National Kidney, 2012).

Hemodynamic pathways include elevations of both systemic and intraglomerular pressure and activation of various vasoactive hormone pathways, including the renin-angiotensin aldosterone system (RAAS), endothelin, and urotensin. RAAS can regulate blood pressure homeostasis. Renin converts angiotensinogen to AngI, which is further converted by angiotensin-converting enzyme to AngII. The latter is the key promoter of vascular damage, particularly in insulin-resistance conditions. While insulin and its homologous autocrine/paracrine peptide insulin like growth factor -1 (IGF-1) can stimulate NO production, a process mediated by PI3K/AKT signaling pathway, AngII, via its ANG type 1 receptor (AT1R), inhibits the action of insulin on vascular and skeletal muscle (Sowers, 2004). This leads to decrease in NO production in endothelial cells, increased vasoconstriction in vascular smooth muscle cells and reduced skeletal muscle glucose transport (Sowers, 2004, Sowers et al., 2001, Steinberg et al., 1996). Additionally, AngII antagonizes the insulin-induced increase of GLUT-4 transport to the skeletal muscle cell membrane, thereby reducing cellular glucose uptake (Sowers, 2004). The generation of ROS appears to be one of the mechanisms by which AngII interferes with insulin and IGF-1 signaling in these tissues.

The altered hemodynamic pathways act independently and, in concert with hyperglycemia-activated pathways, stimulate intracellular kinases such as PKC, mitogen-activated protein kinase (MAPK), nuclear transcription factors such as NF-κB and various growth factors including TGF-β1, CTGF and VEGF (Figure 13). These growth factors have been implicated in modulation of the immune system and activation of enzymes involved in glucose metabolism.

Inflammation is an important factor implicated in the pathogenesis of DN. Inflammatory cytokines, mainly IL-1, IL-6, IL-18, as well as TNF-α are involved in this process.

Dyslipidemia has also been pointed out as a pathophysiological factor in the development of DN, as it was found to parallel with diabetic kidney disease (Tolonen et al., 2008). In type I diabetic subjects, low high density cholesterol (HDL) and elevated triglycerides associate with obesity, which has also been linked to DN (Iseki et al., 2004). Hyperinsulinemia also plays a role by increasing glomerular capillary pressure and glomerular hyperfiltration, endothelial dysfunction and increased vascular permeability (Figure 13). Furthermore, insulin resistance not only parallels but also precedes the development of DN in type I diabetes patients (Yip et al., 1993). In combination, these factors increase proteinuria, extracellular matrix accumulation, cell hypertrophy, and apoptosis.

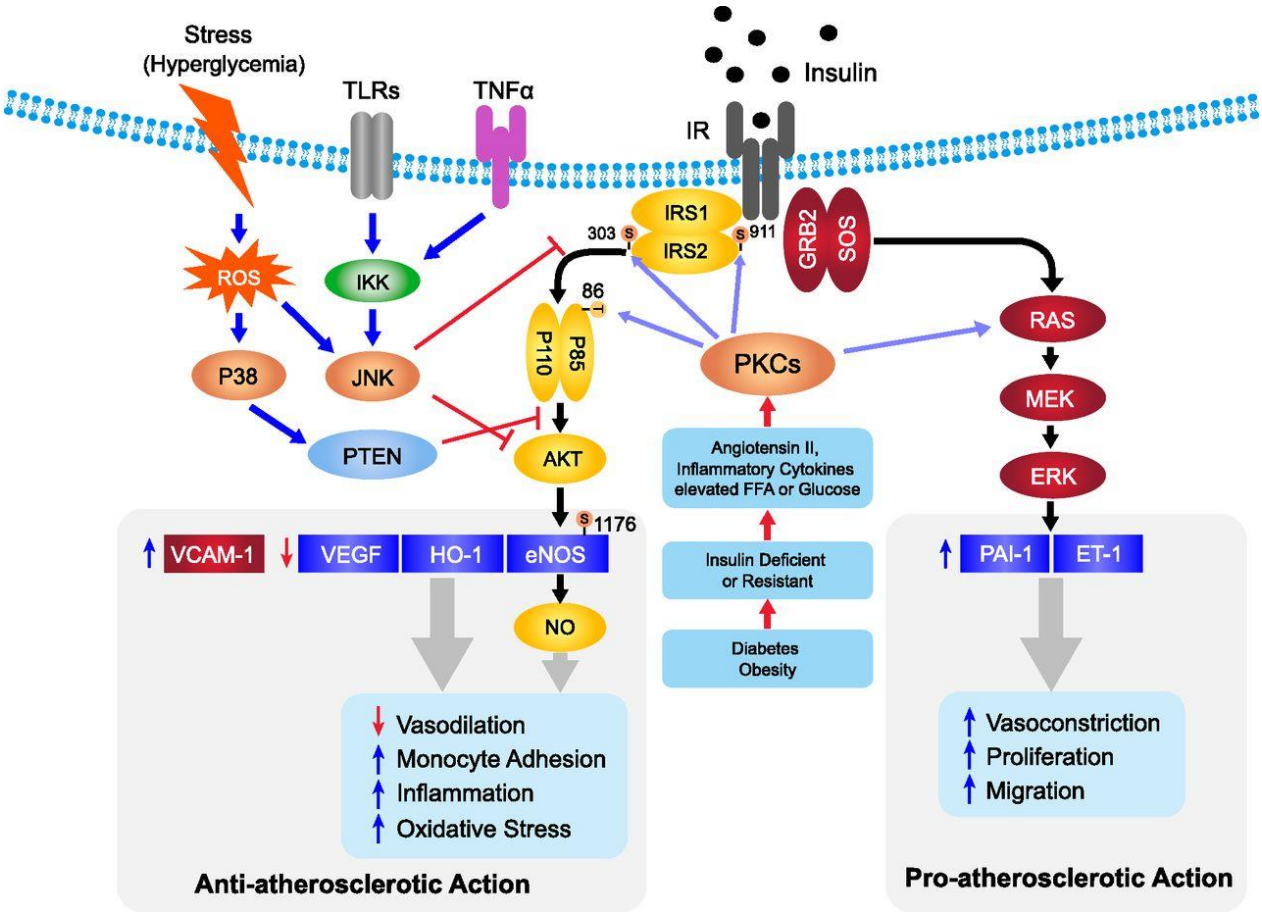

Figure 13. Mechanisms of insulin resistance in vascular endothelial cells. Selective insulin resistance in endothelial cells occurs when AngII, elevated FFA and glucose levels, and proinflammatory cytokines induced by diabetes and insulin resistance stimulate PKC isoforms and other stress kinases to phosphorylate IRS1/2 and PI3K and inhibit only the IRS/PI3K/Akt pathway. In contrast, insulin's stimulation of the SOS/Grb2/MAPK pathway is unaffected or even enhanced. The selective loss of insulin's actions via the IRS/PI3K/Akt pathway causes the reduction of insulin's anti-atherosclerotic action and contributes to the acceleration of atherosclerosis and other cardiovascular pathologies in diabetes. FFA: free fatty acids; PTEN, phosphatase and tensin homolog; IRS, insulin receptor substrate proteins. Adapted from (King et al., 2016).

1.11.3 Oxidative stress in DN

Hyperglycemia causes tissue damage through different mechanisms: increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of AGEs, increased expression of the receptor for advanced glycation end products and its activating ligands, activation of PKC isoforms, overactivity of the hexosamine pathway. Several lines of evidence

indicate that all these mechanisms are activated by a single upstream event (Brownlee, 2005): mitochondrial overproduction of ROS.

The main factor in the production of intracellular ROS is the inability of individual cells to control glucose uptake in hyperglycemic conditions. Generally, cells are able to maintain intracellular glucose homeostasis, by regulating its uptake. However, after chronic exposure to high glucose levels, some cell types lose the ability to control intracellular glucose concentration (Heilig et al., 1995). Indeed, enhanced glucose uptake has been observed in many cell population within the diabetic kidney, including mesangial cells, proximal tubule epithelial cells and podocytes (Coward et al., 2005).

Once glucose accumulates in the cells, it enhances mitochondrial respiratory chain, leading to excess of NADH production, oxidative phosphorylation that promote mitochondrial dysfunction and O_2^- generation.

Free radicals such as superoxide can induce cell and tissue injuries throughout lipid peroxidation, activation of NF-κB (Ha et al., 2008), production of $ONOO^-$, PKC activation and induction of apoptosis. Furthermore, ROS and other free radicals can directly induce injury.

Oxidative stress activates pathogenic pathways such as RAAS, polyol pathway, PKC, increase of some cytokines (IGF-1, TGF-β) and the oxidative stress pathway (Giacco and Brownlee, 2010, Kang et al., 2008, Schena and Gesualdo, 2005, Singh et al., 2014, Vasavada and Agarwal, 2005). Formation of AGEs leads also to production of ROS (Choi et al., 2008, Ha et al., 2008, Vasavada and Agarwal, 2005). AngII activates NADPH oxidase that leads to superoxide formation (Ha et al., 2008, Rodrigo and Bosco, 2006, Vasavada and Agarwal, 2005). AGEs can induce ROS production and activate PKC by induction of oxidative stress in mesangial cell (Stephens et al., 2009).

1.11.4 Genetic contribution to DN development

Although hyperglycemia is the trigger for DN progression, several gene variants confer susceptibility to DN development, i.e. *ACE*, *GLUT*, *IL*, *TNF-α*, *APOE*, *eNOS*, *SOD2*, *COL4A1* etc (Rizvi et al., 2014). New biomarkers associated with renal and cardiovascular outcome have been identified, such as CTGF and osteoprotegerin. CTGF is a key factor in ECM production and other pro-fibrotic activity, that is induced in renal cells upon hyperglycemia and is upregulated in DN. Osteoprotegerin is a secretory glycoprotein belonging to the tumor necrosis factor receptor superfamily that may be involved in the development of vascular calcification (Simonet et al., 1997).

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2. Aims of the thesis

CYGB represents one of the latest additions to the mammalian globin family. CYGB is ubiquitously expressed in a wide variety of organs and occurs mainly in fibroblast-related cells and some neurons of the CNS. Despite more than 10 years of extensive research, the function of CYGB remains unknown, but it has been suggested to be involved in various aspects of oxygen-related metabolism, including ROS detoxification. Moreover, CYGB deficiency has been associated with increased cell death and apoptosis in different cell types as well as *in vivo*. The goal of this project was to expand the existing knowledge on CYGB by focusing on its regulation and function in the kidney. To achieve this goal the specific aims included:

I. Assessment of the anti-oxidative and anti-apoptotic function of CYGB in renal cell models, particularly in podocyte cell lines.

II. To study the association of CYGB expression with chronic kidney diseases (CKD) via independent approaches.

III. The characterization of a CYGB alternative transcript variant (termed CYGB-A1) that has been recently identified in the lab of Thomas Hankeln (University of Mainz, Germany), comparing the expression levels of CYGB and CYGB-A1 in podocyte cell lines under different experimental conditions, including hypoxia and H_2O_2 treatment.

3. Manuscript I: The anti-oxidative role of cytoglobin in podocytes suggests a potential association with chronic kidney disease.

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Running title: CYGB is potentially associated with CKD

Keywords: oxidative stress, reactive oxygen species, ROS, diabetic nephropathy

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ABSTRACT

Cytoglobin (CYGB) is a member of the mammalian globin family, in addition to hemoglobin, myoglobin, neuroglobin and androglobin. Despite extensive research efforts, its physiological role remains largely unknown, but potential functions include reactive oxygen species (ROS) detoxification and signaling. Accumulating evidence suggests that ROS play a crucial role in podocyte detachment and/or apoptosis during diabetic kidney disease. To assess the putative anti-oxidative function of CYGB in podocytes, we stably knocked-down CYGB in the human podocyte cell line AB8/13. CYGB-deficient podocytes displayed an increase in cell death and accumulation of ROS as assessed by H_2-DCF-DA assays and the redox sensitive probe roGFP2-Orp1. Transcriptome analysis of control and CYGB knock-down cells identified dysregulation of multiple genes involved in apoptosis and involved in CKD, particularly in diabetic nephropathy.

INTRODUCTION

Cytoglobin (Cygb) belongs to the superfamily of globins in addition to haemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb) and the recently identified androglobin (Adgb). Most known globins fulfil respiratory functions, supplying the cell with adequate amounts of O_2 for aerobic energy production via the respiratory chain in the mitochondria (Vinogradov and Moens, 2008). In contrast to Mb and Hb, the recently identified mammalian globins Cygb and Ngb (Burmester et al., 2002, Burmester et al., 2000) as well as Adgb (Hoogewijs et al., 2012b) all display hexacoordination of the heme iron atom (i.e. bound by two amino acid residues of the globin fold). While no functional interpretation for heme hexacoordination currently exists, it is thought to point to a role other than classical Hb- and Mb-like oxygen delivery. The expression profile of CYGB has been extensively studied (Hankeln et al., 2005). CYGB is predominantly expressed in fibroblasts and related cell types, but also in distinct nerve cell populations. Its function is less characterized; potentially CYGB may have a role in oxygen storage, NO metabolism, ROS protection or signalling. Mimura et al. (2010) and Nishi et al. (2011) have provided indications for an antifibrotic role of CYGB in the kidney using CYGB-overexpressing transgenic rats, potentially via a ROS scavenging function. Furthermore, Li et al. (2012) have suggested that CYGB could play a protective function under ischemic conditions by reducing nitrite to NO. Lately, several lines of evidence were provided for a NO dioxygenase function of CYGB *in vitro* (Gardner et al., 2010, Liu et al., 2012, Tejero and Gladwin, 2014). Singh et al. (2014) suggested a major role of CYGB in muscle repair and regeneration as myogenic progenitor cells derived from a newly generated mouse model, in which CYGB was specifically knocked out in skeletal muscle, were severely deficient in their ability to form myotubes. Moreover, Thuy et al. (2015) confirmed the antioxidant role of CYGB using a global knock-out mouse model which displays age-dependent development of multiple organ abnormalities. The pathophysiological role of Cygb in kidneys remains poorly understood.

Glomerular visceral epithelial cells, namely podocytes, are highly specialized cells that actively participate in the glomerular filtration process due to their foot processes and slit diaphragm. Podocyte injury is the primary cause of impaired glomerular filtration, which leads to protein leaking as observed in diabetic nephropathy (DN). Because DN is clinically characterized by proteinuria and pathologically by glomerular hypertrophy and GBM thickening with foot process effacement, podocytes have been the focus in DN research. DN is a progressive and irreversible renal disease characterized by the accumulation of extracellular matrix in glomerular mesangium and kidney interstitial tissue which eventually leads to renal failure (Kang et al., 2008). Several mechanisms have been suggested to be involved in the pathogenesis of DN and its complications,

all of them originating from hyperglycemia. Accumulating evidence exists that oxidative stress plays a key role in most pathogenic pathways of diabetic complications (Gnudi, 2012). Free radicals such as superoxide can induce cell and tissue injuries through lipid peroxidation, activation of NF-κB (Ha et al., 2008), production of peroxynitrite and induction of apoptosis. Furthermore, ROS and other free radicals can directly induce injury. *In vitro* studies indicated that CYGB is able to scavenge free radicals, and overexpression of CYGB in various cell lines preserves cell viability under conditions of oxidative stress (Fordel et al., 2006, Hodges et al., 2008, Li et al., 2007, Stagner et al., 2009, Xu et al., 2006), but its role in podocytes remains largely unexplored.

In the current study, we investigated the putative anti-oxidative role of CYGB in human kidney-derived podocyte cell lines. Podocytes lacking CYGB displayed increased ROS accumulation and cell death, altered expression of genes involved in the antioxidant defense system, apoptosis and senescence. Moreover, human renal biopsy expression data from CKD patients indicates an association of CYGB and DN and suggests a putative protective role of CYGB in DN.

MATERIALS AND METHODS

Reagents

Antimycin A (AMA; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 95% ethanol. H_2O_2 was purchased from Sigma-Aldrich. 2'7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (10 μM; Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) was dissolved in DMSO. *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (Sigma-Aldrich) for MTT assays was dissolved in PBS.

Cell culture and treatments

Conditionally immortalized human podocyte cell lines AB8/13, LY, AK, K30, and ATC were a kind gift from A. Kistler (Saleem et al., 2002). Cells were cultured in RPMI (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Thermo Fischer Scientific), 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS; Roche, Mannheim, Germany). AB8/13 cells were propagated at 33°C and differentiated for 10-14 days at 37°C in a humidified incubator at 5% CO_2 (35.7 mmHg) (Binder, Tuttlingen, Germany).

mRNA and protein detection and quantification

Total cellular RNA was extracted as previously described (Stiehl et al., 2012). Total RNA (2 µg) was reverse transcribed (RT) using AffinityScript reverse transcriptase (Agilent, Santa Clara, CA, USA) and complementary DNA (cDNA) levels were estimated by quantitative polymerase chain reaction (qPCR) using the primers listed in Supplementary Table 1 and a SYBR Green qPCR reagent kit (Sigma- Aldrich) in a MX3000P light cycler (Agilent). Transcript levels were calculated by comparison with a calibrated standard and expressed as ratios relative to ribosomal protein L28 mRNA levels. Immunoblotting, signal imaging and quantification were performed as previously reported (Schörg et al., 2015). Membranes were probed with antibodies against CYGB (EPR13198, Abcam, Cambridge, UK), Synaptopodin (SYNPO) (P-19, SC-21537, Santa Cruz Biotechnology, Dallas, TX, USA), PARP (Cell Signaling Technologies, Danvers, MA, USA), SP-1 (SC-59, Santa Cruz Biotechnology), α-tubulin (Cell Signaling) and β-actin (Sigma-Aldrich). Signals from HRP-coupled secondary antibodies were detected with ECL substrate (Pierce, Thermo Fisher Scientific) using a luminescent image analyzer (LAS-4000, FUJIFILM). β-actin was used as loading control for combined cytoplasmic and nuclear extracts, SP-1 and α-tubulin were used as loading controls for nuclear and cytoplasmic extracts, respectively.

Generation of stable knock-down cell lines

Expression vectors encoding short hairpin RNA (shRNA) sequences targeting human CYGB in a pLKO.1-puro plasmid were purchased from Sigma-Aldrich (shCYGB-1: order number TRCN0000059378; shCYGB-2: order number TRCN0000059381). Control cells (shCTR) were transfected with a non-targeting control shRNA under the control of a U6 promoter in a pKLO.1 puromycin resistance vector (Sigma-Aldrich) as described previously (Schörg et al., 2015). Viral particles were produced in HEK293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and pVSV-G (2.8 µg, all from Invitrogen) using polyethylenimine (PEI) transfection as described before (Stiehl et al., 2012). Cells were transduced with lentiviral-pseudotyped particles and cell pools were cultured with the appropriate antibiotic for selection.

H_2DCFDA assay

AB8/13 cells were seeded in 96-well plates at 80% confluency and incubated with 10 µM H_2DCFDA for 30 min in the dark. Subsequently, specific treatments were performed and fluorescence was measured using a 96-well fluorescence photometer (Infinite 200Pro, Tecan, Männedorf, Switzerland). Analysis was performed using the internal software i9 control.

roGFP2-Orp1 measurements

AB8/13 cells were grown on glass bottom dishes (Ibidi, GmbH, Martinsried, Germany) and transduced with the H_2O_2-sensitive probe roGFP2-Orp1 (Gutscher et al., 2009, Morgan et al., 2011), which was subcloned using the Gateway technology into the pAd/CMV/V5-DEST adenoviral vector (Invitrogen). After 48 h, the oxidation of the sensor was measured at basal level or upon AMA treatment (50 µg/ml, 30 minutes). The live measurements of roGFP2 oxidation and calculations of the degree of oxidation were performed as previously described (Tsachaki et al., 2015).

Cell viability assay

Cell death was measured by the Vi-Cell XR 2.03 Cell Viability Analyzer (Beckman Coulter, Krefeld, Germany) using the trypan blue dye exclusion method. For the MTT assay, AB8/13 were seeded in 96-well flat bottom plates and then exposed to AMA or H_2O_2 at the indicated concentrations (final volume 0.1 ml per well). After 6 h, 10 µl of 5 mg/ml MTT solution in PBS were added to each well for 2 h. Following removal of the medium, 100 µl of DMSO were added to each well to dissolve

the formazan crystals. The absorbance at 540 nm was determined in triplicates using a plate reader (Infinite 200Pro, Tecan) and normalized by non-treated cells.

TUNEL assay

Cells were seeded on coverslips and treated with 250 μM H_2O_2 for 3 h. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of nuclei was performed by using APO-BrdU *in situ* DNA fragmentation assay kit (K401-60; Biovision, Milpitas, CA, USA) following the manufacturer's protocol.

Analysis of human renal biopsies

Human renal biopsy specimens and Affymetrix microarray expression data (HG-U133 Plus2.0 Array) were obtained within the framework of the European Renal cDNA Bank–Kröner-Fresenius Biopsy Bank (Cohen et al., 2002). Diagnostic renal biopsies were obtained from patients after informed consent and with approval of the local ethics committees. Following renal biopsy, the tissue was transferred to RNase inhibitor and microdissected into glomerular (Glom) and tubulointerstitial (Tub) compartments. The microarray expression data used in this study came from individual patients with diabetic nephropathy (DN, Glom (n = 7), Tub (n = 7), focal segmental glomerulosclerosis (FSGS, Glom (n = 16), Tub (n = 7)), rapidly progressive glomerulonephritis (RPGN, Glom (n = 23), Tub (n = 21)) as well as pre-transplant biopsies from living renal allograft donors as controls (LD, Glom (n = 18), Tub (n = 18)). Total RNA was isolated from microdissected glomeruli and tubulointerstitium, reverse transcribed, and linearly amplified according to a protocol previously reported (Cohen et al., 2006) (Cohen et al., 2006). Fragmentation, hybridization, staining and imaging were performed following the Affymetrix expression analysis technical manual (Affymetrix, Santa Clara, CA, USA). For microarray analysis, Robust Multichip Analysis (RMA) was performed. Following normalized RMA, significance analysis of microarrays was conducted using a q-value of <5% to identify genes that were differently regulated between the analyzed groups (Tusher et al., 2001) (Tusher et al., 2001). RT-qPCR validation of renal biopsies was performed as reported earlier (Cohen et al., 2002, Schörg et al., 2015). Pre-developed TaqMan reagents were used for human CYGB (NM_134268) and transcript levels were normalized to 18S rRNA levels (Applied Biosystems, Waltham, MA, USA).

Genome wide association study

Genetic association testing for urinary albumin-to-creatinine ratio (UACR) and meta analysis was performed in the CKDGen and CARe cohorts of European ancestry, with further follow-up genetic analysis of significant SNPs in CARe cohorts of African-American ancestry and in the Diabetes

Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study, as previously described (Boger et al., 2011).

RNA quality control, library construction and RNA sequencing

RNA from three independent samples of shCTR and shCYGB-1 was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Prior to library construction, starting total RNA quality was assessed using Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

RNA Integrity Number (RIN) values ranged from 8.5 to 9.7 indicating high quality and integrity of the RNA samples. RNA was quantified using Qubit RNA HS Assay Kit (Invitrogen).

Libraries were prepared starting from 800 ng of total RNA using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina) including a poly-A selection step following the manufacturer's instructions and sequenced as 2 x 150 nt paired-end reads using Illumina NextSeq 500™ (Illumina). Raw sequences were pre-processed using FASTX-Toolkit 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Pre-processed reads were mapped against the annotated human genome version *hg38* using CLC Genomics Workbench 8.5.1 allowing only one (single) or up to 10 (multiple) mappings for each read using the parameters reported in Table 2. For each dataset, the mapped reads were imported as BAM files in Genomatix Genome Analyzer v3.40820 to perform Principal Component Analysis (PCA) using default parameters. Differentially expressed genes were determined using the EdgeR-based Empirical analysis of DGE (Robinson et al., 2010, Robinson and Smyth, 2008) statistical tool of CLC Genomics Workbench using default parameters. Genes were considered differentially expressed when presenting |fold change| > 2 and false discovery rate (FDR)-corrected p-value ≤ 0.05. In this study, multiple mapping reads were considered.

Statistical analyses

If not otherwise indicated, results are presented as mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using Student's t-test and one-way ANOVA or two-way ANOVA where appropriate. p-values <0.05 were considered statistically significant.

RESULTS

CYGB expression in podocyte cell models

To investigate the renal role of CYGB we explored CYGB expression levels in multiple human kidney-derived cell lines, including proximal tubule cells, fibroblasts, embryonic cells and podocytes. Because most abundant endogenous CYGB expression levels were observed in podocytes, particularly in AB8/13 (Fig. 1A), we employed this cell model for further investigations. The heat-sensitive AB8/13 cells grow at the permissive temperature of 33°C and stop proliferating and undergo differentiation within two weeks at 37°C (Saleem et al., 2002). RT-qPCR revealed that CYGB mRNA is differently expressed before and after podocyte differentiation (Fig. 1B) and is mainly localized in the cytoplasm, as assessed by immunoblotting (Fig. 1C).

The expression of CYGB was evaluated also during mouse kidney embryogenesis (Supplementary Fig. S1). A pronounced upregulation at postnatal stages P14 - P28 was observed, suggesting a putative role of CYGB in kidney development.

Because CYGB expression is increased upon hypoxia in multiple cell lines and organs, including the kidney (Nishi et al., 2011), we assessed whether CYGB was upregulated upon hypoxic treatment in our own cell model. Unexpectedly, CYGB expression was not increased in AB8/13 cells after 24 h in 0.2% O_2 (Supplementary Fig. S2A). Conversely, CYGB mRNA expression in kidneys from mice kept under hypoxia for 12 h was also upregulated (Supplementary Fig. S2B), confirming the earlier observations. To study the role of CYGB in podocytes, we established stable knock-down cells using two independent shRNA sequences targeting CYGB, termed shCYGB-1 and -2. The efficiency of the knock-down in AB8/13 cells was confirmed by analysis at both mRNA and protein levels (Fig. 2A-C).

Anti-apoptotic role of CYGB in AB8/13

DN is characterized by early loss of podocytes due to detachment and/or cell death. A putative anti-apoptotic role of CYGB has been suggested in various cell models (Singh et al., 2014, Tian et al., 2013), but not in podocytes. This prompted us to investigate cell viability in our cell models. CYGB knock-down podocytes displayed profoundly reduced viability (Fig. 2C), indicating a role of CYGB in cell survival. A similar consistent reduction in cell viability was observed in the independent podocyte cell line LY (Supplementary Fig. S3). Consistently, substantially increased cleaved-PARP levels could be observed in shCYGB-1 cells (Fig. 2D). Moreover, MTT assays demonstrated that shCYGB-1 cells are more susceptible to H_2O_2 and AMA compared to WT and shCTR cells (Fig. 3E). TUNEL assays further demonstrated the presence of double-stranded DNA

breaks in shCYGB-1 cells treated with H_2O_2 for 3 h, but not in shCTR cells (Fig. 3F). Overall, these data indicate that CYGB is a mediator of podocyte viability under oxidative stress conditions.

Antioxidative role of CYGB in AB8/13

To investigate the putative anti-oxidative role of CYGB in podocytes, we treated AB8/13 WT cells with 250 μM H_2O_2 and measured CYGB mRNA levels at different time points. CYGB expression was induced after 1 and 2 h and tended to decrease within time (Fig. 3A). The well-established anti-oxidant gene HO-1 (heme oxygenase-1) was used as a positive control. Interestingly, basal expression levels of redox-sensitive genes HO-1 and HSP1A1 were increased in shCYGB-1 cells compared to WT and shCTR cells, indicating an CYGB-dependent altered intracellular redox state (Fig. 3B). To further confirm this observation, WT, shCTR and shCYGB cells were transiently transfected with the redox-sensitive GFP-probe roGFP2-Orp1 which is sensitive to cytoplasmic oxidation. Compared to WT cells, shCYGB showed a significant increase in roGFP2-Orp1 oxidation under basal conditions (white bars), consistent with the gene expression data. Additionally, treatment with antimycin A (AMA), an inhibitor of mitochondrial electron transfer at complex III, further increased roGFP2-Orp1 oxidation (black bars), particularly in the two shCYGB cell models. (Fig. 3A). A fluorescent cell-permeable indicator of ROS (H_2DCFDA) was used as an independent approach to measure ROS generation in podocytes upon different stimuli. WT podocytes and experimental cells were plated in equal numbers in 96-well plate. After 14 days of differentiation, podocytes were incubated with 10 μM of H_2DCFDA for 30 minutes and subsequently exposed to AMA, or H_2O_2 (Fig. 3B, C). Treatment with 50 μg/ml AMA or H_2O_2 increased ROS production in WT, shCTR and more pronouncedly in shCYGB-1 cell lines (Fig. 3D-E), particularly in shCYGB-1. Additionally, treatment with high glucose concentrations (HG) significantly increased ROS accumulation (Fig.3G) and cell death (Fig. 3H) in shCYGB cells compared to WT, indicating a protective role of CYGB in oxidative-stress mediated cell death. On the other hand, overexpression of CYGB reduced ROS accumulation and oxidative stress as assessed by roGFP2-Orp1 oxidation and H_2DCFDA based assays upon different stimuli (Suppl. Fig. S4). Collectively, these data provide evidence for an anti-oxidative role of CYGB in podocyte cell lines.

Transcriptome analysis of CYGB deficient cells revealed altered expression of multiple genes involved in redox signaling and apoptosis.

To study the CYGB-dependent transcriptome we performed RNA sequencing analysis on shCTR and shCYGB-1. Three independent samples for each condition were analysed and the knock-

down efficiency of shCYGB-1 was confirmed by RPKM (Reads per Kilobase per Million mapped reads) of multiple mapping transcriptome analysis, which results in > 90% CYGB downregulation (Fig. 4A). Most of the total reads mapped into exons (90%), whereas introns (9%) and intergenic regions (1%) count only for 9% and 1% of the total reads, respectively (Fig.4B), confirming the high quality of the multiple mapping. More than 1500 genes were found to be differentially expressed in shCYGB-1 compared to shCTR (Fig. 4D-E) and among these, the majority (1148) were downregulated. Several anti-oxidant genes, such as DUOX-1, DUOX-2, GPX-3, as well as some anti-apoptotic genes, like TP73, were found to be downregulated, whereas genes involved in podocyte injury, such as SERPINE1 and CTGF, were upregulated. The most relevant differentially expressed genes are summarized in Table 3 and were validated by RT-qPCR on independent RNA samples (Fig. 4F).

CYGB is potentially associated with CKD

Genome-wide association studies (GWAS) have proven to be a very powerful tool in identifying novel genetic markers involved in CKD (O'Seaghdha and Fox, 2011). By using GWAS databases (Borger et al 2011) we identified a single-nucleotide polymorphism (SNP), located in an intergenic region 3' of the CYGB gene potentially associated to albuminuria, although with marginal significance (Fig. 5A) (Boger et al., 2011). *UCSC*-integrated *ENCODE* data illustrate that the SNP (rs8082416) is localized in a DNaseI hypersensitivity cluster that reflects open chromatin, and overlaps with strong transcription factor occupancy (Supplementary Fig. S4). ChIP-sequencing datasets further revealed histone marks for an active enhancer (H3K4Me1) and basal components of the transcriptional machinery (RNA pol2 and p300) in this region. Finally, RNA polymerase 2-associated ChIA-PET (chromatin interaction analysis by paired-end tag sequencing) data suggests DNA looping of this potential enhancer region to the transcription initiation site of the CYGB gene. As a fully independent approach, a comprehensive analysis of existing gene array data from human renal biopsy specimens collected from the European Renal cDNA Bank was performed (Cohen et al., 2002). Data were obtained from Affymetrix HG-U133 Plus 2.0 microarrays, hybridized with glomerular and tubulointerstitial cDNA procured from different nephropathies as well as pretransplant biopsies from living renal allograft donors as controls. These biopsy expression studies from CKD patients revealed a consistent association of CYGB expression levels with advanced end-stage CKD, including diabetic nephropathy (Fig. 5B). These CYGB mRNA expression data in different kidney diseases were validated by RT-qPCR in an independent patient cohort, supporting the association of increased CYGB mRNA levels with

diabetic nephropathy (Fig. 5C). In summary, these data obtained by 2 independent methodologies, illustrate an association of CYGB with CKD.

DISCUSSION

This study demonstrates that CYGB can protect podocytes from oxidative stress and cell death in different experimental conditions. Particularly, CYGB deficient podocytes display increased ROS accumulation and cell death, activation of pro-apoptotic pathway and dysregulation of genes involved in cellular redox balance, apoptosis and podocyte integrity. Additionally, we provided evidence for the putative association of CYGB with CKD, particularly with DN.

The anti-oxidative function of CYGB has reached a consensus in the globin field, however the precise molecular mechanism underlying the anti-oxidative function is still poorly understood. Most likely CYGB may scavenge ROS using heme and thiol residues (Fordel et al., 2007a, Petersen et al., 2008), while peroxidase and superoxide dismutase activity have been excluded due to the lower tissue concentration of CYGB compared to known enzymes with similar role as well as by the investigations of Trandafir et al. (Trandafir et al., 2007).

In the present study, we investigated for the first time the role of CYGB in kidney cell models, particularly in podocytes, which express higher levels of CYGB than other kidney-derived cell lines. The generation of stable CYGB knock-down cell lines, using two independent shRNA sequences and the validation in an independent podocyte cell model (Suppl. Fig. S3) strengthen our findings. CYGB deficiency was associated to increased cell death and DNA damage (Fig. 2), consistent with the previously observed anti-apoptotic role of CYGB (Fordel et al., 2007b, Latina et al., 2015, Singh et al., 2014, Stagner et al., 2009). CYGB overexpression reduces oxidative stress, prevents ROS accumulation in H_2O_2 or AMA treated-podocytes and promote cell viability, in line with previous findings (Fordel et al., 2007b, Hodges et al., 2008, Xu et al., 2006). Additionally, increased ROS production and cell death upon high glucose treatment are more pronounced in CYGB knock-down cells compared to controls. Notably, hyperglycemia-induced ROS accumulation may contribute to podocyte apoptosis and depletion (Liu et al., 2013, Susztak et al., 2006).

Transcriptome analysis of CYGB deficient podocytes and control cells revealed dysregulation of multiple genes involved in redox balance, apoptosis, podocyte function and podocyte injury. Most of the genes were downregulated, indicating a general suppression of the transcriptional process in the absence of CYGB. Due to its homology to Mb and their comparable O_2-binding affinity, CYGB was thought to contribute to the intracellular O_2 supply (Trent and Hargrove, 2002), acting as an O_2 reservoir or as signal transducer in O_2 sensing pathways (Geuens et al., 2003, Hankeln et al., 2005), therefore, CYGB deficiency would decrease oxygen availability and ROS signaling. DUOX-1 and -2 belong to the NADPH oxidases (NOX) family and need oxygen as co-substrate to produce ROS (i.e. H_2O_2 or O_2^-). Both DUOX-1 and -2 have been found to be downregulated in

lung cancer (Luxen et al., 2008) and in hepatocellular carcinoma (DUOX-1) (Ling et al., 2014). However, their role in podocytes is still unknown. Downregulation of anti-oxidative genes, such as CYGB, SOD3, GPX3 and GPX7 could explain the accumulation of ROS, as previously reported (Singh et al., 2014). Intriguingly, SOD3 and GPX3 downregulation occurs in 1 month old CYGB total knock-out mice (Thuy et al., 2015). The tumor suppressor TP73 encodes a member of the p53 family of transcription factors involved in cellular responses to stress and participates in the apoptotic response to DNA damage. Cells from mice lacking this isoform (DeltaNp73) have shown to be more sensitive to DNA-damage with increased p53-dependent apoptosis (Wilhelm et al., 2010).

Lack of CYGB leads to downregulation of podocyte markers involved in maintaining cell differentiation, such as WT-1 and MAFB. WT-1 is a key regulator of podocyte function and its downregulation leads to glomerulonephritis and mesangial sclerosis (Guo et al., 2002).

MAFB is essential for kidney development (Moriguchi et al., 2006) and its overexpression in podocytes protects against DN, through the regulation of slit diaphragm proteins, anti-oxidative enzymes, and the Notch pathway (Morito et al., 2014).

Analysis of murine CYGB expression during mouse kidney development showed a pronounced upregulation at postnatal stage P14 - P28, suggesting a putative role of CYGB in kidney embryogenesis. Additionally, kidney cysts spontaneously occur in 13% of CYGB global knock-out mice within 6 months of age (Thuy le et al., 2016), consistent with a putative protective role of CYGB in the kidney. Mimura and colleagues reported on the anti-fibrotic role of CYGB in kidney (Mimura et al., 2010).

GWAS has proven to be a very powerful tool in identifying novel genetic markers involved in CKD (O'Seaghdha and Fox, 2011). We discovered a SNP in the 3' intergenic region of the *CYGB* gene, potentially associated with increased albuminuria (Fig. 5A). The SNP coincides with a region displaying several hallmarks of an enhancer and loops back to the CYGB transcription initiation site as indicated by ChIA-PET data. Intriguingly, this is the first time that a globin gene was associated with CKD. Simultaneously, the potential role of CYGB in acquired human renal disease was found in an unbiased way by analyzing transcriptomic data of patients with different stages of renal failure. A pronounced upregulation of CYGB was detected in independent DN specimens (Fig. 5B-C). Similarly, CYGB has found to be induced in rat models of chronic nephropathy and pancreatitis (Nakatani et al., 2004). CYGB is upregulated upon hypoxia in a wide variety of organs (Emara et al., 2010, Fordel et al., 2004b, Fordel et al., 2007a, Mammen et al., 2006, Schmidt et al., 2004) and was reported to be induced in fibrotic kidney lesions, which are known to be frequently hypoxic.

In conclusion, our study demonstrates that CYGB (i) is expressed at high levela in human podocyte cell lines, (ii) protects podocytes from oxidative stress and apoptosis, and (iii) may be involved in CKD, particularly in DN.

LIST OF ABBREVIATIONS

AMA = antimycin A
AP-1 = activator protein-1
ARE = antioxidant response element
CA9 = carbonic anhydrase IX
CYGB = cytoglobin
DCF = 2`,7`-dichlorofluorescein
ECM = extracellular matrix
H2DCFDA = 2`,7`-dichlorodihydrofluorescein diacetate
HIF = hypoxia-inducible factor
HO-1 = heme oxygenase 1
ROS = reactive oxygen species
RPKM = reads per kilobase per million mapped reads
ChIA-PET = chromatin interaction analysis by paired-end tag sequencing

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Figure 1

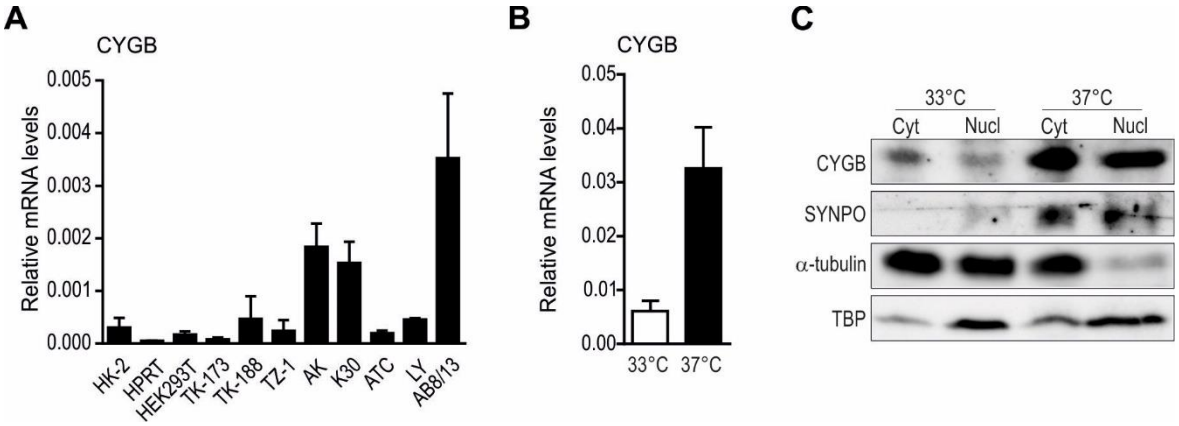


Figure 1. AB8/13 cells express abundant CYGB mRNA levels. A. CYGB mRNA expression in human kidney-derived cell lines. HK-2 and HPRT, proximal tubule cell lines; HEK293T, embryonic kidney; TK-173, TK-188 and TZ-1, fibroblasts; AK, K30, ATC, LY and AB8/13, podocytes. **B.** CYGB mRNA expression and **C.** protein levels in AB8/13 before (33°C) and after 10-14 days of differentiation at 37°C. Cyt, cytoplasmic fraction; Nucl, nuclear fraction.

Figure 2

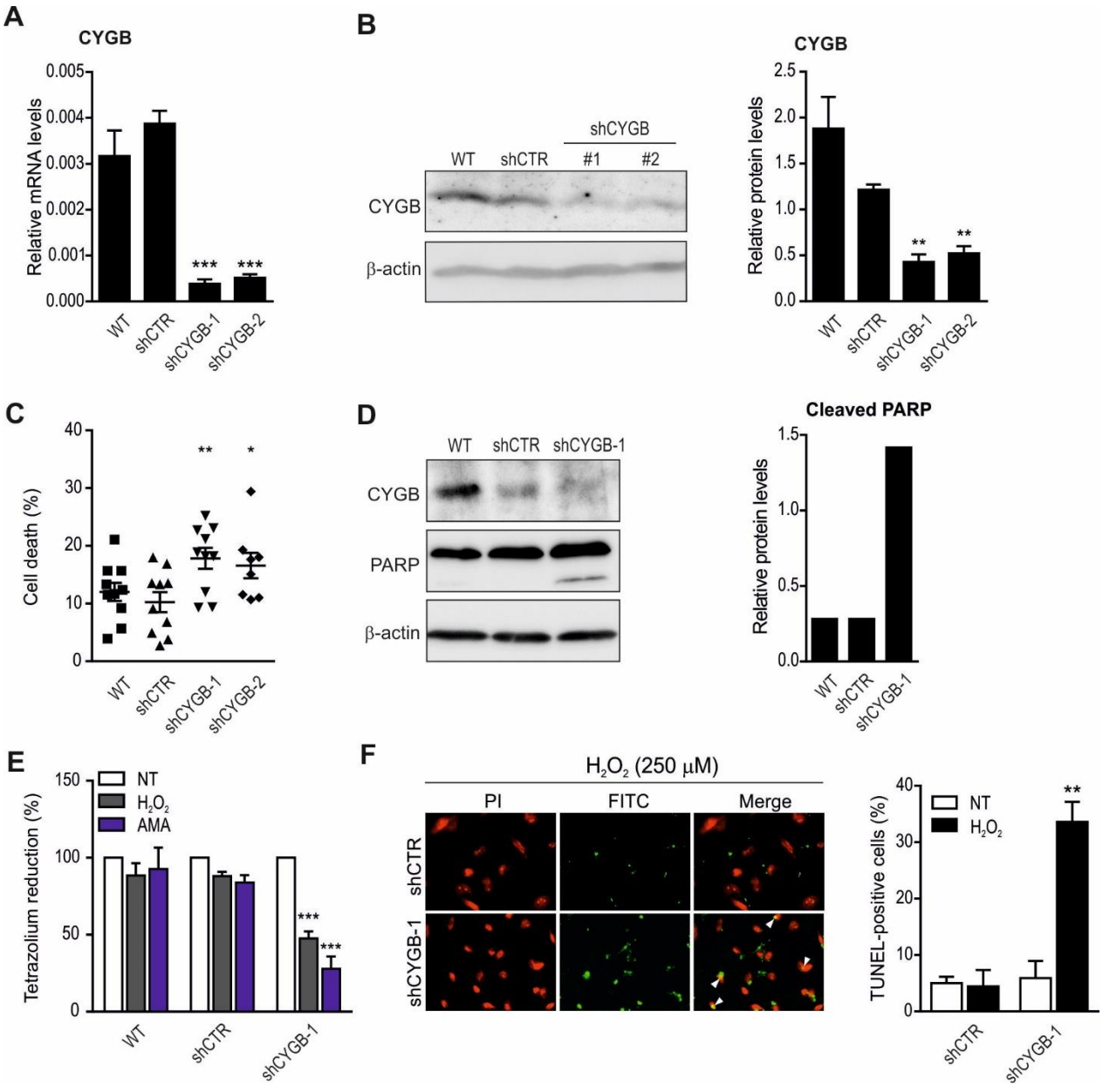


Figure 2. CYGB protects against cell death and apoptosis. A. Stable CYGB knock-down efficiency (shCYGB-1, -2) was confirmed at the mRNA level. ***p<0.001 one-way ANOVA and Tukey correction for multiple comparisons. **B.** Representative immunoblot (left panel) and densitometry analysis (right panel). **p<0.01 one-way ANOVA and Dunnett correction for multiple comparisons to shCTR (control). **C.** Cell death was measured by trypan blue dye exclusion. **p<0.01 one-way ANOVA and Tukey correction for multiple comparisons. **D.** Representative

immunoblot displaying PARP cleavage in CYGB deficient cells compared to WT and shCTR (left panel) and densitometry analyis (right panel). **E.** MTT viability assay showed that shCYGB, WT or shCTR cells after antimycin A (AMA) or H_2O_2 treatment; **p<0.01 one-way ANOVA and Tukey's post hoc test. **F.** TUNEL assay in shCTR and shCYGB-1 cells following H_2O_2 treatment (left panel) and corresponding quantification of TUNEL-positive cells (right panel). Arrowheads indicate TUNEL positive staining. **p<0.01 one-way ANOVA and Tukey correction for multiple comparisons.

Figure 3

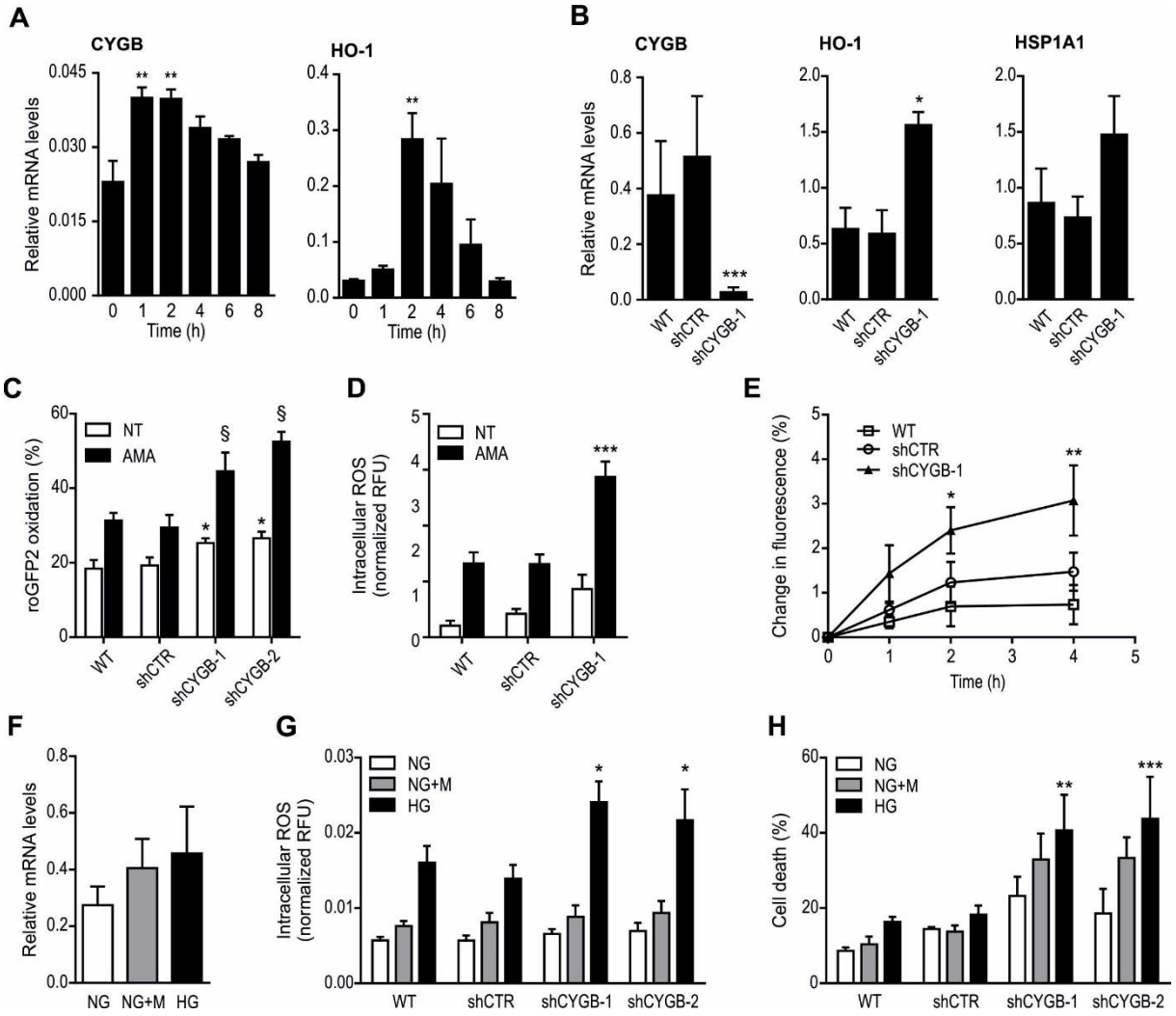


Figure 3. Lack of CYGB is associated with increased oxidative stress under basal conditions and upon different stimuli. A. CYGB expression in AB8/13 WT cells following H_2O_2. The expression of the anti-oxidant gene HO-1 was used as positive control. **B.** Basal expression of the anti-oxidant genes HO-1 and HSP1A1 in WT, shCTR and shCYGB-1 cells. *p<0.05, ***p<0.001 one-way ANOVA and Tukey's post hoc test. **C.** Quantification of the cytoplasmic redox-sensitive probe roGFP2-Orp1 oxidation in WT, shCTR and shCYGB-1 cells under basal conditions (white bars) and upon AMA treatment (black bars). *p<0.05 Student's t test compared to NT WT. §p<0.05 compared to AMA WT. **D.** Intracellular ROS production following AMA (50 μg/ml) treatment. ***p<0.001 two-way ANOVA and Tukey's post hoc test compared to WT and shCTR. **E**. Kinetics of H_2O_2 treatment in WT, shCTR and shCYGB-1 cells. Results were calculated as

increase in fluorescence per well ((Ftx – Ft0) / Ft0*100), where Ftx = fluorescence at a specific time point and Ft0 = fluorescence at 0 h. **F.** CYGB mRNA levels upon 5 days of normal glucose (NG), mannitol (NG+M) and high glucose (HG) treatment. **G.** ROS accumulation in WT, shCTR and shCYGB-1 and -2 cells upon NG, NG+M and HG treatment. **H**. Cell death quantification (trypan blue exclusion method) of WT, shCTR and shCYGB-1 and -2 cells subjected to NG, NG+M and HG for 5 days. NG, (5.6 mM), normal glucose; NG+M, (NG + 24.4 mM mannitol), osmotic control; HG, (30 mM), high glucose. Statistical analyses were performed with two-way ANOVA and Tukey's post hoc test compared to WT HG (*p<0.05; **p<0.01; ***p<0.001).

Figure 4

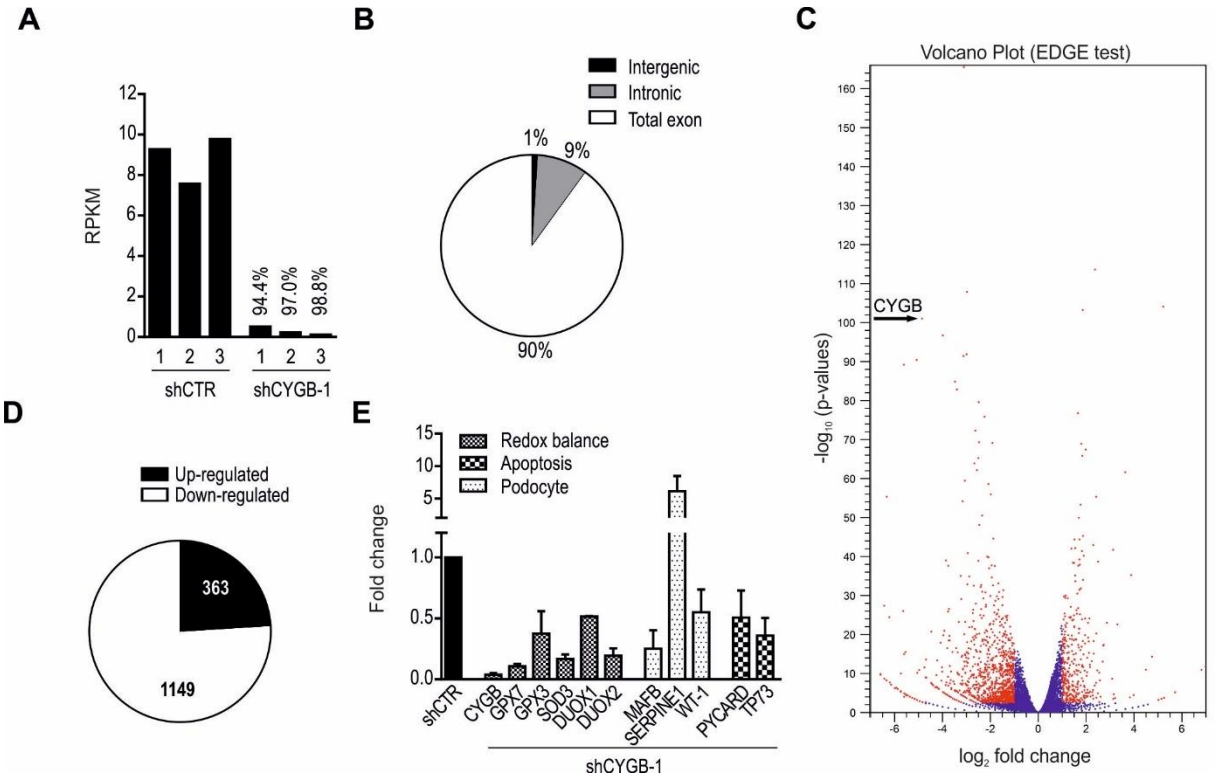


Figure 4. CYGB knock-down alters expression of genes involved in redox balance, apoptosis and podocyte phenotype. A. CYGB gene expression in RPKM (Reads per Kilobase per Million mapped reads) of multiple mapping transcriptome analysis of WT and CYGB knock-down podocytes. **B.** Mapping reads classification into total exons, introns and intergenic regions. **C.** Volcano plot showing the distribution of differentially expressed genes, marked in red. Red dots, |Fold change|>2, p<0.05; blue dots, |Fold change|<2, not significant. **D.** Up-regulated and down-regulated genes from the in total 1511 differentially regulated genes. **E.** Validation of the results by RT-qPCR on independent RNA samples. Values are expressed as fold change compared to shCTR (set to 1).

Table 3: List of differentially expressed genes in shCYGB-1 compared to shCTR. Genes are divided in four categories, anti-oxidant, podocyte phenotype, podocyte injury and apoptosis. P values are corrected by FDR.

Function	Gene ID	Fold change	p-value
Anti-oxidant	**CYGB**	**-28.92**	**1.1E-98**
Anti-oxidant	**SOD3**	**-3.70**	**8.0E-04**
Anti-oxidant	**GPX3**	**-4.69**	**9.97E-19**
Anti-oxidant	**GPX7**	**-2.96**	**4.8E-03**
Anti-oxidant	**DUOX-1**	**-18.14**	**3.3E-05**
Anti-oxidant	**DUOX-2**	**-12.40**	**3.8E-07**
Podocyte phenotype	**MAFB**	**-86.20**	**1.8E-25**
Podocyte phenotype	**WT1**	**-2.26**	**4.1E-02**
Podocyte injury	**SERPINE1**	**2.19**	**1.87E-27**
Apoptosis	**PYCARD**	**-2.73**	**2.7E-06**
Apoptosis	**TP73**	**-2.45**	**2.05E-08**

Figure 5

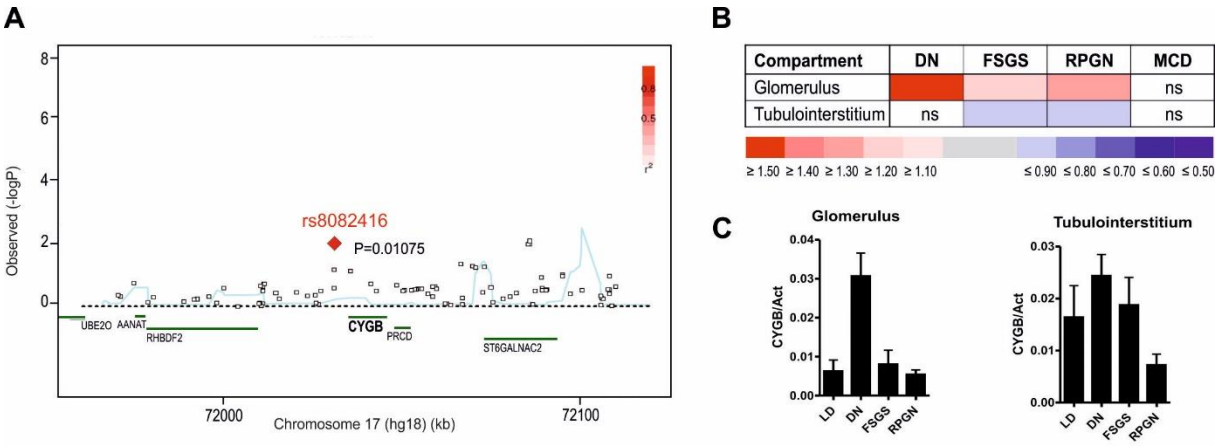


Figure 5. *CYGB* is potentially associated with chronic kidney disease. A. Regional association plot for the *CYGB* gene region displaying a SNP potentially associated with CKD obtained from a genome-wide association study (Boger et al., 2011). Log_{10} P values are plotted versus genomic position, using gene annotations obtained from *UCSC* Genome Browser (RefSeq Genes, build 36). **B.** Gene expression data of microdissected glomeruli and tubulointerstitial compartments from patients with glomerulopathies. Microarray data analysis was performed with the single probe-based analysis tool, ChipInspector. Values are fold changes relative to living donors (LD) for the probe with the highest coverage. DN, diabetic nephropathy; RPGN, rapidly progressive glomerulonephritis; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease. **C.** CYGB mRNA levels were validated by RT-qPCR in an independent cohort of microdissected samples. CYGB mRNA expression levels were normalized to β-actin expression levels.

Supplementary methods

Cells and reagents

The human podocyte cell lines AB8/13 and LY were propagated at 33°C and differentiated for 10-14 days in a 37°C humidified incubator, at 5% CO_2 (35.7 mmHg) (Binder, Tuttlingen, Germany). Hypoxia experiments were carried out at 0.2% oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies) for 24 h.

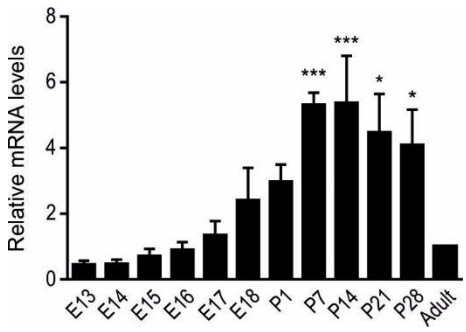
Generation of stably overexpressing cell lines

The full-length human CYGB gene and the control gene beta-glucuronidase (Gus) were cloned into pLENTI6 plasmid. Viral particles were produced in HEK293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and pVSV-G (2.8 µg, all from Invitrogen) using polyethylenimine (PEI) transfection as described before (Stiehl et al., 2012). Cells were transduced with lentiviral-pseudotyped particles and cell pools were cultured with the appropriate antibiotic for selection.

Supplementary Table 1. Primers used for qPCR amplification

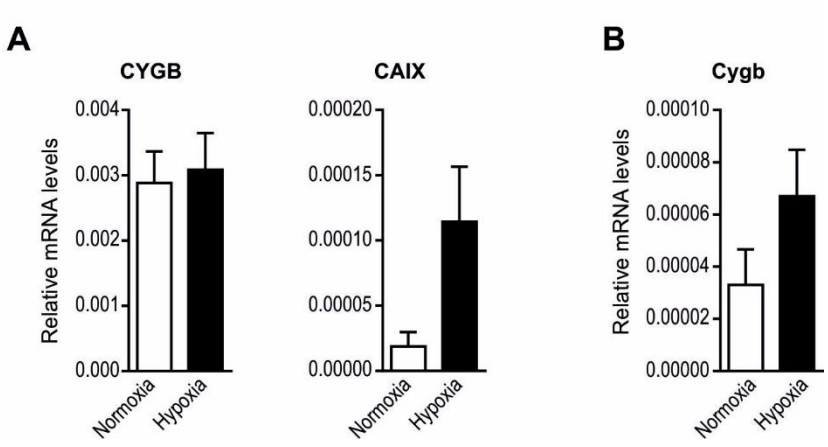
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
CYGB	CAAGGTGGAACCGGTGTACT	TCACGTGGCTGTAGATGAGG	137
DUOX1	CAGCTGGAAGAGGAAAACAAGG	TGCAGAGTGTGTTCCTTAGGC	138
DUOX2	GAAGGCTGTGACAAAGCAGC	AACATGTCCTGGGGCTTGAG	201
GPX3	AACTCCTGTCCTCCCACCTC	ATCTTGACGTTGCTGACCGT	167
GPX7	GCAGGAGCAGGACTTCTACG	CTCGGTAGTGCTGGTCTGTG	137
HO-1	ATGACACCAAGGACCAGAGC	GTGTAAGGACCCATCGGAGA	153
HSP1A1	TGCTGATCCAGGTGTACGAG	CGTTGGTGATGGTGATCTTG	204
L28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198
MAFB	TCGACCTGCTCAAGTTCGAC	AGTTGCTCGCCATCCAGTAC	204
PYCARD	GCCGAGGAGCTCAAGAAGTT	ATAAAGTGCAGGCCTGGCTT	296
SERPINE1	GACCTCAGGAAGCCCCTAGA	ACTGTTCCTGTGGGGTTGTG	275
SOD3	CGAGACATGTACGCCAAGGT	AACTGGTGCACGTGGATGG	248
TP73	CGAAAATGCCAACAAACGGC	AGATTGAACTGGGCCGTGG	247
WT-1	GCGGAGCCCAATACAGAATA	TCTCACCAGTGTGCTTCCTG	207

Supplementary Figure S1



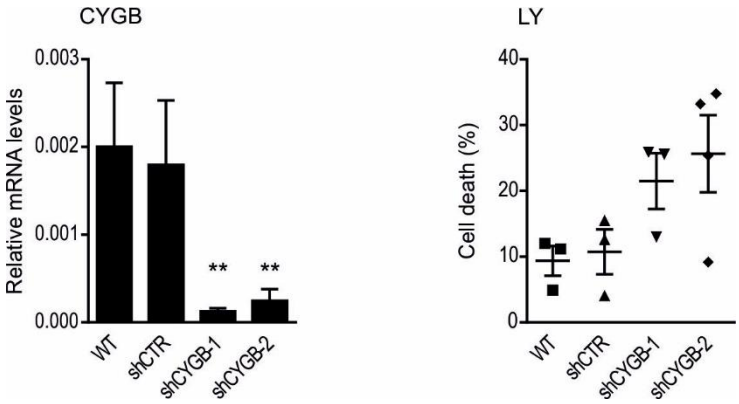
Supplementary Figure S1. Temporal renal expression profile of CYGB. CYGB expression increases during mouse kidney development and subsequently decreases during adulthood (n=5). Statistical analyses were performed with one-way ANOVA and Dunnett's multiple comparisons test compared to adult (*p<0.05; ***p<0.001).

Supplementary Figure S2



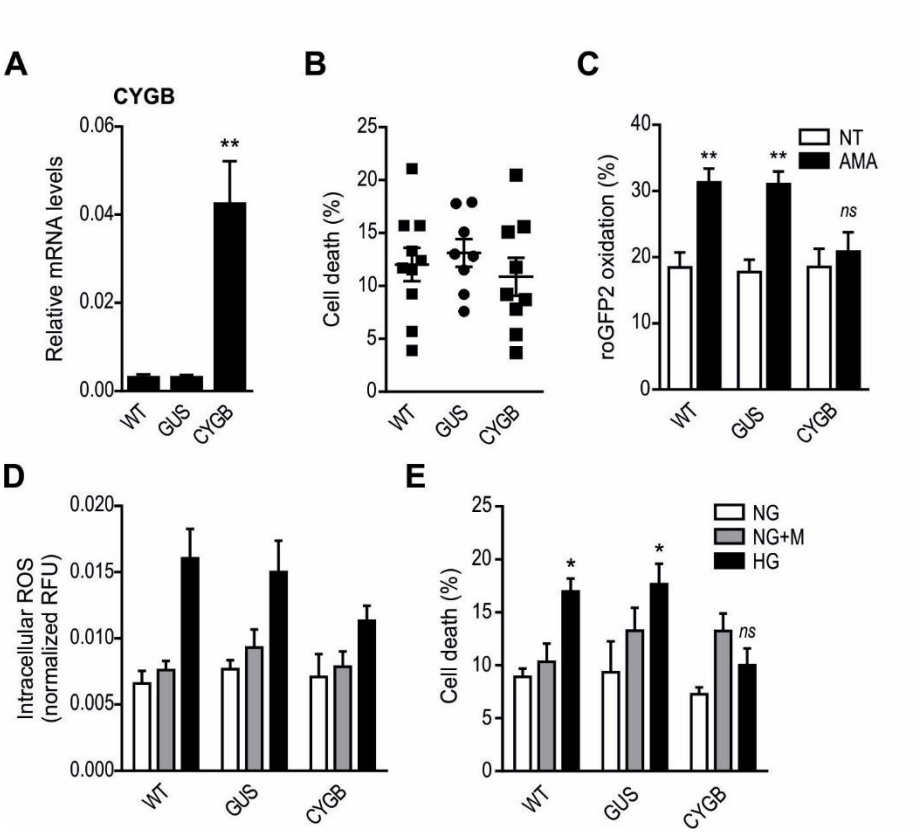
Supplementary Figure S2. CYGB expression upon hypoxia in AB8/13 and *in vivo*. A. CYGB expression levels in AB8/13 exposed to 21% O_2 (normoxia) or 0.2% O_2 (hypoxia) for 24 hours. CAIX was used as hypoxic positive control. **B**. CYGB mRNA levels were determined by RT-qPCR in kidneys from mice kept under normoxia or hypoxia (8% O_2) for 12 hours and normalized to ribosomal S12 RNA.

Supplementary Figure S3



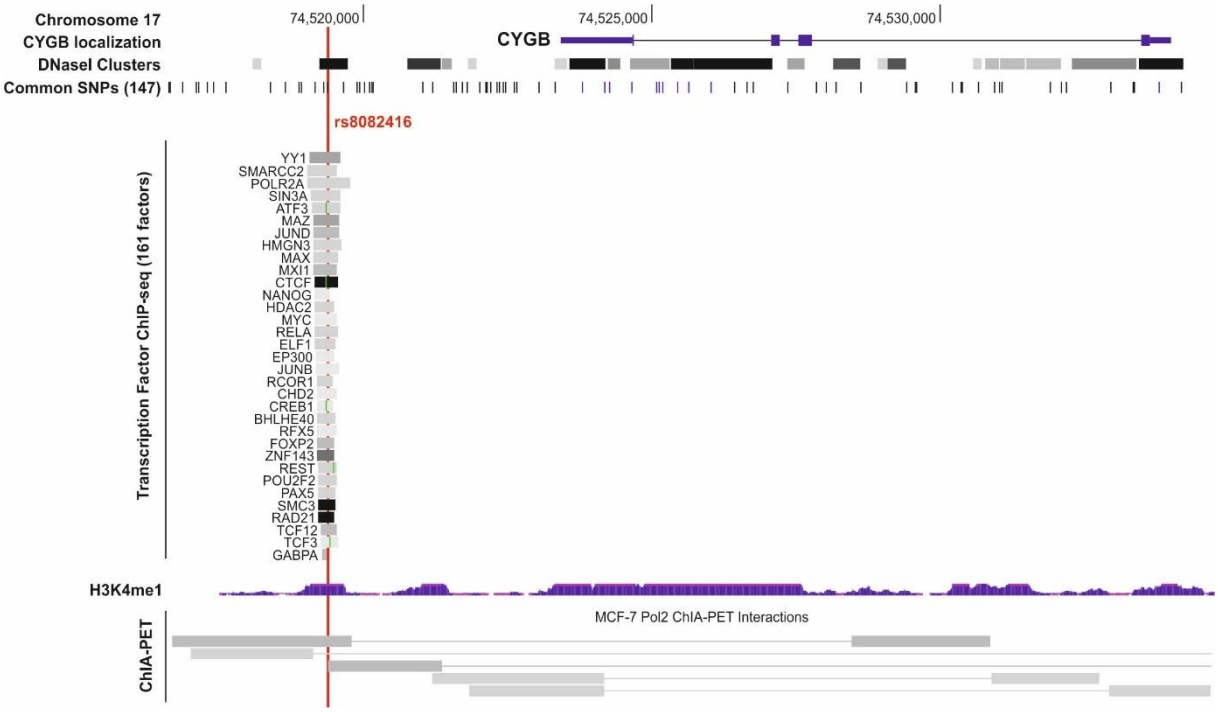
Supplementary Figure S3. Generation of CYGB stable knock-down in the independent podocyte cell line LY. A. Stable CYGB knock-down in LY cells was confirmed by RT-qPCR. **B**. Cell death was measured by trypan blue dye exclusion.

Supplementary Figure S4



Supplementary Figure S4: CYGB overexpression protects podocytes against ROS accumulation and cell death. A. Efficacy of CYGB overexpression in AB8/13 cells. **B.** Cell death measured by trypan blue exclusion. **C.** Quantification of roGFP2 oxidation at basal level and after antimycin A (AMA) treatment, showing no significant difference in CYGB non-treated (NT) and treated samples. **D.** Measurement of intracellular ROS accumulation by H_2DCFDA treatment, and **E.** corresponding cell death upon normal (NG) or high glucose treatment (HG). Mannitol (NG+M) was used as osmotic control. GUS overexpression (GUS) was used as control for CYGB overexpression (CYGB).

Supplementary Figure S5



Supplementary Figure S5. CYGB SNP rs8082416 coincides with a DNaseI hypersensitivity cluster and strong transcription factor occupancy. UCSC genome browser output (*hg19*) of the SNP rs8082416 (red) and CYGB gene locus revealed that the SNP is located in the 3' intergenic region of CYGB and localized in a DNaseI hypersensitivity cluster with substantial transcription factor occupancy. Additionally, ChIA-PET interaction studies suggest loop formation between the SNP area and the transcription initiation region of the CYGB gene.

4. Manuscript II: Identification of an alternative cytoglobin transcript variant (unpublished results)

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Running title: Cytoglobin transcript variant characterization

Keywords: CYGB isoform, alternative first exon, ROS

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ABSTRACT

The human genome uses alternative pre-mRNA splicing as an important mechanism to encode a complex proteome from a relatively small number of genes. An unknown number of these genes also possess multiple transcriptional promoters and alternative first exons that contribute another layer of complexity to gene expression mechanisms. We have recently identified an isoform of cytoglobin (CYGB), termed CYGB-A1, that presents an alternative first exon, located 10 kb upstream of the canonical one. CYGB belongs to the mammalian globin family, in addition to hemoglobin, myoglobin, neuroglobin and androglobin. The function of CYGB remains largely unknown, but accumulating evidence suggests its involvement in ROS scavenging. Additionally, CYGB has a tumor suppressor function in several cancers. In this study, we compared the basal expression of CYGB and CYGB-A1 in different cell models. Moreover, CYGB and CYGB-A1 mRNA levels were quantified following various experimental conditions, including hypoxia and ROS induction. *In silico* analysis of the promoter region of the alternative isoform showed the binding of numerous transcription factors and a different methylation status compared to the canonical first exon.

INTRODUCTION

Alternative splicing and alternative promoter usage are effective mechanisms for generating diverse gene products from the same genomic region. Recent high-throughput analysis has demonstrated that the majority of human genes are subjected to transcriptional variations (Kampa et al., 2004). Thereby, alternative splicing and transcription appear to be the major regulators of post-transcriptional modifications and may cause a significant change in the function of the encoded protein. By inactivating tumor suppressors or by a gain-of-function in proteins that promote abnormal cell growth, alteration of transcription may be involved in tumorigenesis. Additionally, another recent genome-wide analysis has revealed the presence of a large number of alternative promoters (Carninci et al., 2006). Alternative transcription initiation may regulate tissue- or time-dependent expression due to a different response to transcription factors or by epigenetic changes. Moreover, it may regulate protein function by variations that could be generated in the N-terminal region (Hughes, 2006). The alternative transcripts variants are translated into protein isoforms with different biological properties that could affect protein:protein interaction, subcellular localization or catalytic ability (Davis et al., 1992, English et al., 1995, Kestler et al., 1995).

Expressed sequence tag (EST) and cDNA sequence evidence (Harrow et al., 2012), microarray data (Sanchez-Pla et al., 2012) as well as RNA-seq data (Uhlen et al., 2015) are currently employed to detect splicing and alternative transcription events. In recent years, the number of alternative transcripts in the reference database *GENCODE* human gene set (v24) has drastically increased, counting 82 141 coding sequence (CDS) distinct protein-coding transcripts (Harrow et al., 2012). EST data comparison strongly indicates that similar levels of alternative splicing occur in distinct species, such as human, mouse, *Drosophila* and *C. elegans*, emphasizing the importance of alternative splicing throughout evolution (Brett et al., 2002).

Bioinformatic analysis of EST tracks and independent RNA-seq data led to the identification of 5 alternative transcript variants of human cytoglobin (CYGB). In this study, we focused on the comparison between the canonical transcript CYGB and a transcript variant termed CYGB-A1 that shows an alternative first exon and lacks the A and B helices. The two transcripts display different transcriptional regulation and respond to ROS stimuli in a distinct manner.

MATERIALS AND METHODS

Cell culture and reagents

The conditionally-immortalized human podocyte cell lines (AB8/13, AK, LY, K30, ATC) were cultured in RPMI (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen), 5 µg/ml insulin-transferrin-sodium selenite (ITS; Roche, Mannheim, Germany). Human podocyte cell lines were propagated at 33°C and differentiated for 10-14 days in a 37°C humidified incubator at 5% CO_2 (35.7 mmHg) (Binder, Tuttlingen, Germany). Hypoxia experiments were carried out at the indicated concentration of O_2 and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies) as previously described. H_2O_2 (Sigma-Aldrich) and antimycin A (AMA; Sigma-Aldrich) were used to induce ROS at the indicated time points.

RNA extraction and quantitative PCR

RNA was extracted by using the phenol-chloroform method (Stiehl et al., 2012) and cDNA was generated by reverse transcription (RT) of 2 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in combination with a MX3000P light cycler (Agilent) and the primers listed in Supplementary Table 1. Initial template concentrations were calculated by comparison with serial dilutions of a calibrated standard. Ribosomal protein L28 mRNA levels were used to normalize the data.

Statistical analyses

If not otherwise indicated, results are presented as mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using Student's t-test and one-way ANOVA or two-way ANOVA where appropriate. P-values <0.05 were considered statistically significant.

RESULTS

Identification of CYGB transcript variants

In silico analysis of EST sequences revealed the presence of distinct transcripts, summarized in Table 1. The canonical transcript CYGB (Figure 1, black) consists of four exons, spanning 2158 base pairs and encodes a protein of 190 amino acids. The transcript ID, mRNA length and the predicted number of protein amino acids of all variants are summarized in Table 2. Figure 1 displays the *UCSC* genome browser output of the exon composition and localization of the alternative transcripts. Interestingly, CYGB-A1 (Figure 1, red) contains an alternative first exon, located 10 kb upstream of the canonical one. This results in a globin lacking the A and B helices, which are usually translated by the canonical first exon. Because CYGB-A1 was first identified in RNA-sequencing data by one of us, E.P., we focused our investigation on the comparison between the original transcript CYGB and CYGB-A1.

CYGB and CYGB-A1 are differentially regulated in normoxia and hypoxia in podocyte cell lines

We previously demonstrated that CYGB is abundantly expressed in the podocyte cell line AB8/13 (Randi et al. in preparation). We investigated the expression levels of CYGB and CYGB-A1 in various podocyte cell lines, comparing non-differentiated vs differentiated cells and normoxic vs hypoxic conditions. CYGB expression is increased upon hypoxia at both mRNA and protein levels in a wide variety of organs (Fordel et al., 2004b, Fordel et al., 2007a, Mammen et al., 2006, Schmidt et al., 2004), including kidney, eyes, brain, heart, liver, skeletal muscle and in different cellular models (Fordel et al., 2004b, Guo et al., 2007, Singh et al., 2009). CYGB and CYGB-A1 display different absolute expression levels in five independent podocyte cell lines (Fig. 2A-B).
In the AB8/13 cell model, CYGB-A1 is 2-fold less expressed compared to CYGB. In contrast to the hypoxic control CAIX, CYGB-A1 is not upregulated upon hypoxia in any of the cell lines, whereas CYGB is induced in AK, K30 and ATC. As AB8/13 displayed the highest absolute CYGB-A1 expression levels we employed this cell model for further investigations.

CYGB and CYGB-A1 are differentially regulated following H_2O_2 or AMA treatment.

CYGB was found to be upregulated in response to oxidative stress, i.e. after H_2O_2 treatment in breast cancer and neuroblastoma cell lines (Chua et al., 2009, Li et al., 2007). To investigate the effect of ROS on CYGB-A1 expression, we treated AB8/13 cells with H_2O_2 or AMA for different periods. HO-1 is known to be strongly upregulated by oxidative stress and was used as positive

control. Following H_2O_2 treatment, CYGB displayed slight upregulation, whereas CYGB-A1 was pronouncedly induced (Fig. 3A). The kinetic of the response of CYGB and CYGB-A1, as well as HO-1, was comparable, with the highest peak after 2 h. The mRNA levels of CYGB, CYGB-A1 and HO-1 decreased to normal levels after 8 h, most likely due to the potential decay of the H_2O_2 (Fig. 3A). While CYGB was not induced by AMA treatment, CYGB-A1 showed an upregulation (Fig. 3B), suggesting that its expression is more sensitive to experimental ROS induction.

DISCUSSION

Alternative splicing and alternative transcription are mechanisms by which organisms increase the biodiversity of proteins that can be encoded by the genome (Modrek and Lee, 2002). In this study, we provided a preliminary characterization of the expression and the function of an alternative transcript variant of cytoglobin (CYGB-A1), recently identified in a RNA sequencing study.

Cytoglobin (CYGB) was discovered in 2001 (Kawada et al., 2001) and was subsequently classified as a member of the mammalian globin family (Burmester et al., 2002). Several lines of evidence suggest that CYGB could have an anti-oxidative function, preserving cell viability (Li et al., 2007, Singh et al., 2014, Thuy le et al., 2015).

We recently identified several alternative transcript variants of CYGB which display distinct characteristics (summarized in Table 2). The most interesting transcript variant was named CYGB-A1 and displays an alternative first exon located 10 kb upstream the canonical one. The use of an alternative first exon leads to a protein product that lacks the A and B helices.

First attempts to express CYGB-A1 recombinantly in bacteria remained unsuccessful. Due to the absence of the A and B helices, the protein might show structure misfolding. Globins display a highly conserved 3D structure, with a hydrophobic core that surrounds the heme group and hydrophilic surfaces that maintain globin solubility (Lesk and Chothia, 1980). The absence of A and B helices and, therefore, of important amino acid residues for helical interactions may alter the hydrophobic/hydrophilic properties of the globin and generate unstable globin molecules. Crystallography studies show that CYGB is a homodimer (de Sanctis et al., 2003, Sugimoto et al., 2004) stabilized by hydrogen bonds, electrostatic interactions (de Sanctis et al., 2004a) and two intramolecular disulfide (S–S) bridges between Cys38(B2) of one monomer and Cys83(E9) of another monomer, and *vice versa* (Sugimoto et al., 2004). However, CYGB can also be monomeric when heme is at micromolar concentrations (Lechauve et al., 2010). In the monomeric form, the two Cys residues might form an intramolecular S–S bond (Hamdane et al., 2003, Lechauve et al., 2010). In both monomeric or dimeric forms of CYGB, Cys38(B2) and Cys83(E9) are crucial for protein stability and might be involved in the ligand binding process (Sugimoto et al., 2004), by promoting conformational changes. Therefore, absence of the B helix in CYGB-A1 prevents S-S bridges formation and could reduce CYGB-A1 stability. Additionally, S–S bond in CYGB can be influenced by cellular redox state, affecting ligand binding abilities (Hamdane et al., 2003). We can speculate that a monomer of CYGB-A1 could interact with a monomer of CYGB, leading to a formation of a heterodimer with modulated O_2 affinity properties.

We analyzed the expression of the canonical transcript CYGB and CYGB-A1 in different podocyte cell lines and upon various stimuli (Fig. 2-3). In AB8/13, CYGB-A1 displayed lower basal

expression levels compared to CYGB, but was highly induced upon treatment with H_2O_2 or AMA (Fig. 3). The distinct expression regulation suggests the involvement of different promoters and regulatory regions modulated potentially by other transcriptional factors. The hypothesis that different promoters regulate the transcription of *CYGB-A1* and allow the influence of various transcriptional regulators resulting in a tissue and signal specific expression pattern remains to be further investigated. Transcripts that present distinct promoters are often tissue and/or developmental specific (Wang et al., 1999), resulting in effects restricted to certain cells or developmental stages. This could explain the differential expression of the two transcripts in undifferentiated and differentiated cells (Fig. 2).

In conclusion, we identified an alternative transcript variant of CYGB that shows an alternative first exon. CYGB-A1 is differentially regulated under basal conditions and upon hypoxic and oxidative stress, suggesting its possible contribution as anti-oxidative protein. Although the physiological relevance of CYGB-A1 remains to be further explored, our study adds another layer of complexity to CYGB gene expression mechanisms.

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FIGURES

Table 1. Alternative transcript variants of CYGB. *(predicted)

Name	Transcript ID	bp	Protein length*	Biotype
CYGB-001 (CYGB)	ENST00000293230.5	2158	190 aa	Protein coding
CYGB-002	ENST00000589342.1	815	226 aa	Protein coding
CYGB-003	ENST00000590175.1	830	125 aa	Protein coding
CYGB-004	ENST00000586160.1	842	No protein	Processed transcript
CYGB-005 (CYGB-A1)	ENST00000589145.1	788	125 aa	Protein coding

Figure 1

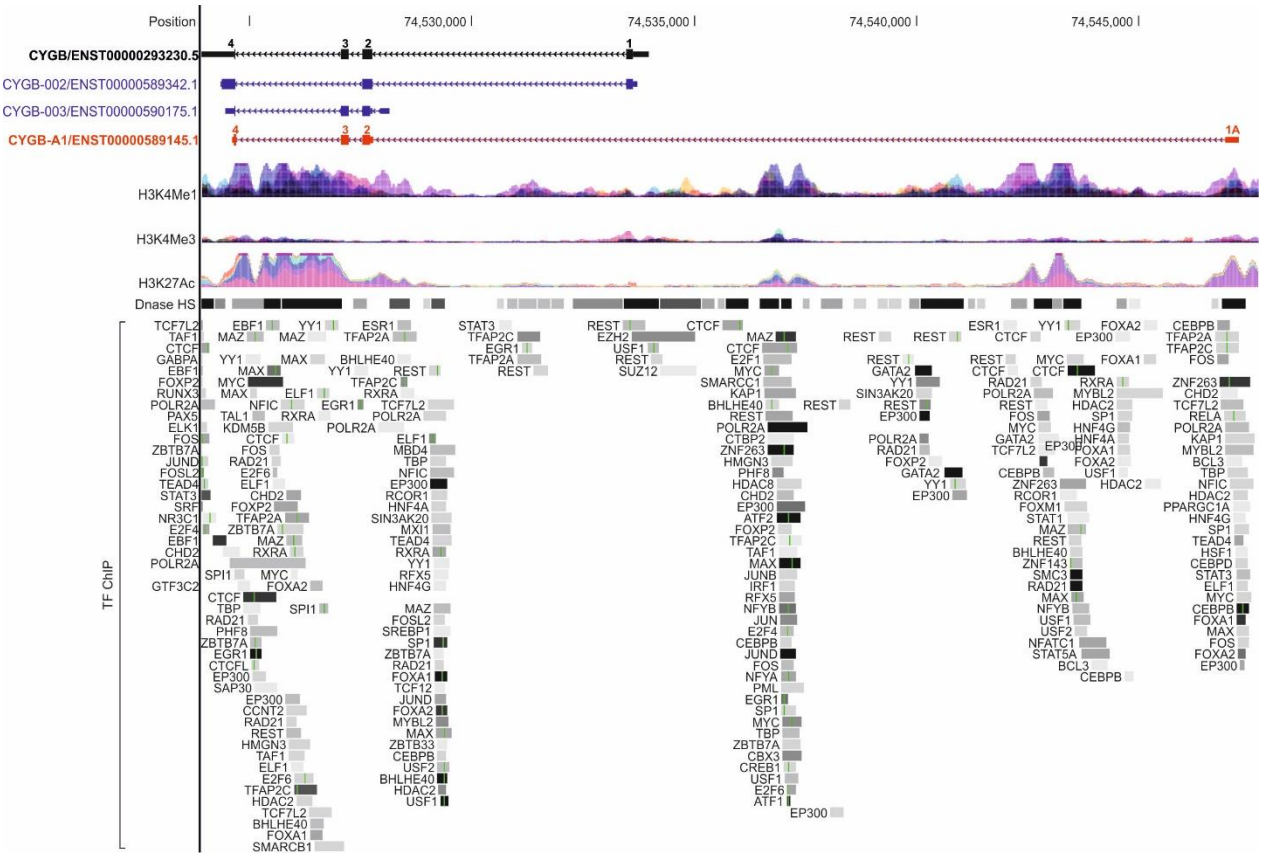

Figure 1. *UCSC* Genome Browser output (*hg19*) of the *CYGB* genomic locus and the transcript variants on chromosome 17. *CYGB* is depicted in black, *CYGB-A1* is in red, whereas the other alternative transcripts (CYGB-002 and -003) are in blue. CYGB-004 is not shown. The number of the exons for *CYGB* and *CYGB-A1* are shown. Methylation (H3K4Me1, Me3) and acetylation (H3K27Ac) profiles are displayed. DNase I hypersensitivity clusters (DNAse HS) and the transcription factors are shown (TF ChIP). A1, alternative exon 1.

Figure 2

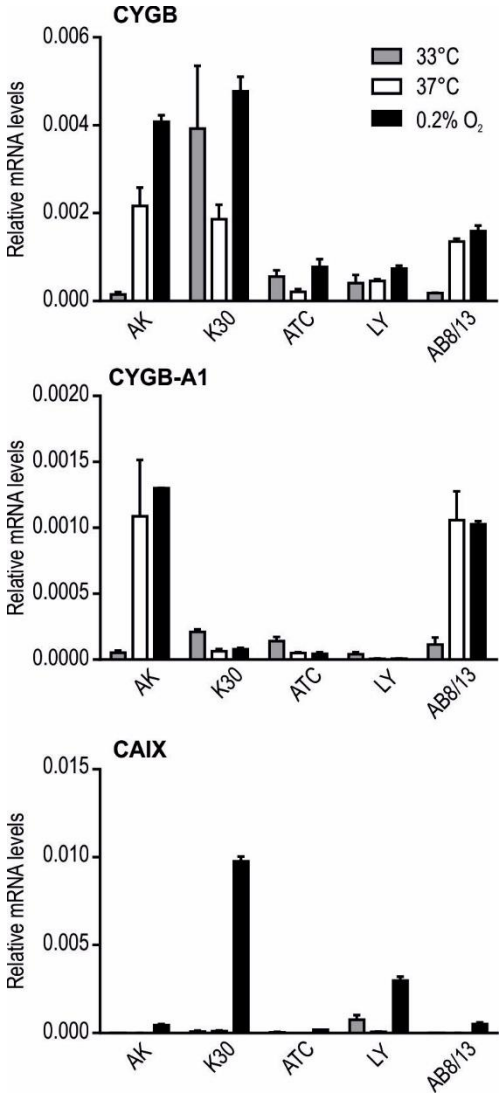

Figure 2. CYGB and CYGB-A1 are differentially expressed in various podocyte cell lines. Expression of CYGB and CYGB-A1 in human podocyte cell lines at basal level in non-differentiated (33°C) and differentiated (37°C) conditions and upon hypoxic treatment (0.2% O_2) for 24 h in differentiated cells. CAIX was used as positive control for hypoxia.

Figure 3

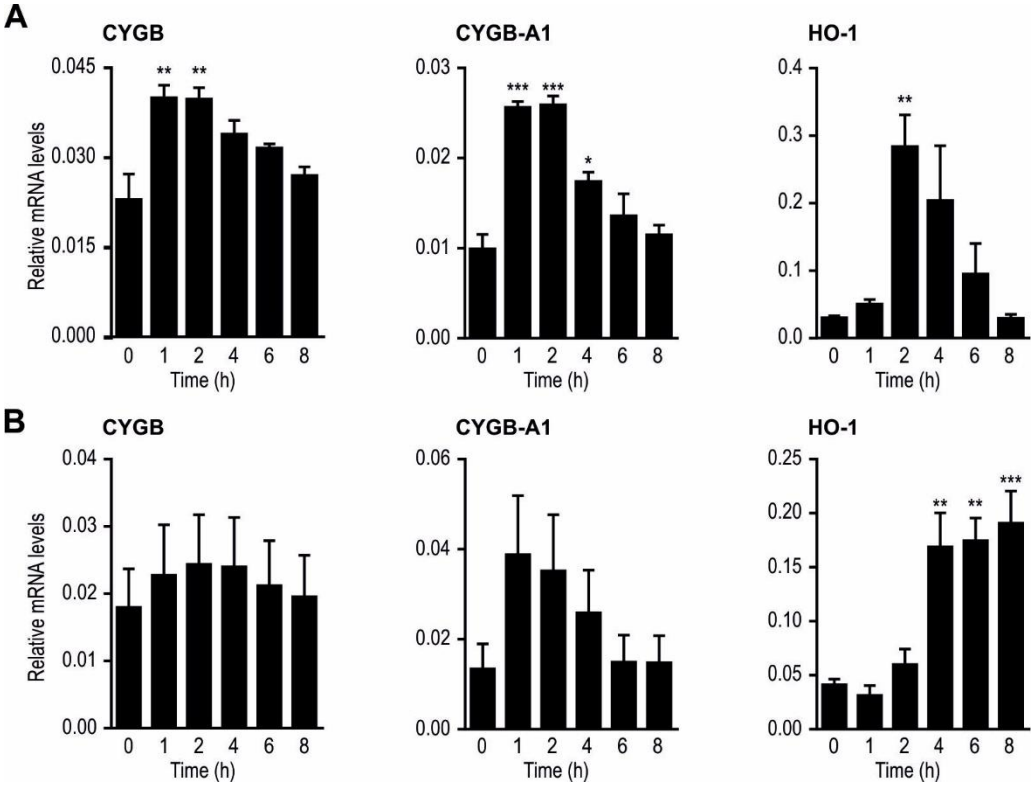

Figure 3. CYGB-A1 displays different regulation compared to CYGB upon H_2O_2 and AMA treatment. A. AB8/13 cells were treated with H_2O_2 (250 μM) or **B.** AMA (50 μg/ml) for the indicated time points. The ROS-inducible HO-1 gene was used as positive control for the treatments.

Supplementary Table 1. Primers used for qPCR amplification.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
CYGB	CAAGGTGGAACCGGTGTACT	TCACGTGGCTGTAGATGAGG	137
CYGB-A1	AACATCACGGGACTTCGTTAAATCA	ACTGGCTGAAGTACTGCTTGGC	105
HO-1	ATGACACCAAGGACCAGAGC	GTGTAAGGACCCATCGGAGA	153
HSP1A1	TGCTGATCCAGGTGTACGAG	CGTTGGTGATGGTGATCTTG	204
L28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198

5. Conclusions and future perspectives

Cytoglobin (CYGB) belongs to the mammalian globin family, in addition to neuroglobin and androglobin and the well-studied hemoglobin and myoglobin. Despite extensive research efforts, its physiological role remains essentially unknown, but several studies reported on its putative role in ROS detoxification. ROS are detrimental for many disease, including diabetic nephropathy (DN), a common chronic complication of diabetes. Hyperglycemia-induced ROS is responsible for glomerular injuries, such as podocyte apoptosis and/or detachment, which contribute to development of proteinuria.

The aim of the thesis was to investigate the (patho)physiological role of CYGB in podocytes *in vitro*, mainly focusing on its putative anti-oxidative function and its possible association with kidney disease.

Anti-oxidative function of CYGB in podocyte cell lines and putative association with CKD

CYGB was discovered in 2001 as marker of hepatic stellate cells (Kawada et al., 2001) and has been subsequently included in the mammalian globin family. CYGB is ubiquitously expressed in a wide variety of organs and tissues, including liver, brain and kidney and has been suggested to be involved in oxygen sensing and storage, NO scavenging, oxidative stress and hypoxia. While the main target molecule of CYGB remains unknown, the most relevant function of CYGB is likely related to the radical-scavenging ability, as assessed by several studies (Fordel et al., 2007, Hodges et al., 2008, Mimura et al., 2010, Singh et al., 2014, Thuy le et al., 2015, Xu et al., 2006). Currently, most of our knowledge on CYGB function derives from Kawada's group, which focuses its studies mainly on the liver. However, little is known about the physiologic role of CYGB in other tissue. The most recent work on $Cygb^{-/-}$ mice demonstrated that lack of CYGB is associated with multiple organ abnormalities, specifically in liver and kidney. Additionally, Mimura and colleagues suggested a putative anti-fibrotic role of CYGB in the kidney (Mimura et al., 2010).

In our study, we investigated the role of CYGB in kidney cell models, specifically in podocytes, which express high endogenous levels of CYGB relative to other kidney-derived cell lines. Accumulating evidence indicates that podocytes represent one of the main targets of ROS at the onset of DN. To investigate the putative anti-oxidative role of CYGB in podocytes, we established stable CYGB knock-down and overexpressing cell lines and we compared them in terms of cell viability, ROS accumulation and gene expression. CYGB deficiency was associated with increased cell death and DNA damage, consistent with the previously observed anti-apoptotic role of CYGB (Fordel et al., 2007, Latina et al., 2015). Cygb overexpression reduces oxidative stress, prevents ROS accumulation in H_2O_2, AMA or high glucose treated-podocytes, and promotes cell

viability, in line with previous findings (Fordel et al., 2007, Hodges et al., 2008, Xu et al., 2006). To test whether the antioxidant response pathways play a role in CYGB-mediated ROS protection additional experiments may be performed, including e.g. luciferase assays to specifically monitor the activity of Nrf1 and Nrf2. Particularly, ARE (antioxidant response element) and ATF6 (activating transcript factor 6, a member of AP-1 transcription factor dimers) responsive luciferase reporter genes could be employed, using Nrf2 overexpressing plasmid as a positive control.

Transcriptome analysis of CYGB deficient podocytes and control cells was an appropriate tool to obtain further insight into the differentially expressed genes in the presence or absence of CYGB, with the possibility to identify both novel transcriptional regulators and putative downstream targets of CYGB. We observed dysregulation of multiple genes involved in redox balance (i.e. SOD3, GPX3, GPX7, DUOX1, DUOX2), apoptosis (PYCARD, TP73), podocyte function (MAFB, WT-1) and podocyte injury (SERPINE1). Most of the genes were downregulated, indicating a general suppression of the transcriptional process in the absence of CYGB.

Our study provides evidence of a putative CYGB association with chronic kidney disease (CKD) via two independent approaches: GWAS and gene array data from CKD patients. GWAS indicated that the SNP rs8082416 is potentially associated with increased albuminuria, a common manifestation of CKD. Analysis of the *ENCODE* data (pre-release) from the *UCSC* Genome Browser revealed that the SNP colocalizes with a DNase I hypersensitivity cluster, and ChIP-seq data displayed a variety of transcription factors binding to this site. Moreover, ChIA-PET interaction analysis showed the possible physical association between the SNP region and the *CYGB* promoter region, especially for the O_2-regulated variant CYGB-A1. To reproduce if the SNP potentially associates with CKD, luciferase reporter genes could be performed using the endogenous *CYGB* promoter and mutating the 3' intergenic enhancer region of CYGB in a variety of renal cell lines using site-directed mutagenesis. Additionally, a refined analysis of a high density SNP map of the *CYGB* genomic locus from patients with CKD could eventually identify new SNPs specifically associated to DN.

In order to validate our findings *in vivo*, streptozotocin (STZ)-induced DN in $Cygb^{-/-}$ mice has been initiated in collaboration with Sylvia Dewilde (University of Antwerp). WT and $Cygb^{-/-}$ mice have been injected with STZ and blood glucose has been monitored to confirm diabetes development. Four months after the treatment, urine has been collected in metabolic cages, and the principal urine parameters, i.e. glycosuria, proteinuria and glomerular filtration rate, are currently under investigation. At the macroscopic level no obvious differences were found in kidneys from WT and $Cygb^{-/-}$ mice, but further analyses are needed to investigate eventual differences at the microscopic level. Immunohistochemical analyses would reveal eventual morphological changes,

presence of fibrosis, inflammation, apoptosis and differences in podocyte density, as marker of DN.

Identification of an alternative variant of CYGB

The identification of CYGB transcript variants with unknown physiological functions added further complexity to our understanding of *CYGB* transcriptional regulation. Among five distinct transcripts, we focused on the isoform that displays an alternative first exon located 10 kb upstream of the canonical one, termed CYGB-A1 to discriminate it from the main transcript CYGB. The use of an alternative first exon leads to a protein product that lacks the A and B helices.
First attempts to express CYGB-A1 recombinantly in bacteria failed, possibly due to misfolding of the protein in the absence of A and B helices. Transcript variants that display different first exons are also regulated by distinct promoters and transcription factors and consequently, their tissue and signal expression pattern could be different, i.e. being restricted to a specific organ or stage of development. To identify the transcriptional regulators of the transcript variant an *in silico* promoter analysis has been performed. The subsequent validation by chromatin immunoprecipitation (ChIP) and luciferase reporter genes based assays using the endogenous *CYGB* promoter should be performed. Interestingly, alternative variants could also be associated with disease (Tazi et al., 2009). Therefore, the expression pattern of CYGB-A1 should be evaluated in cancer and normal tissues as well as various cell lines. Notably, the lack of high quality antibodies against CYGB still represents a limitation to finally solve its distribution pattern in specific tissue. The renal expression profile of CYGB remains still poorly investigated, although two independent groups reported on CYGB expression in mouse glomerular cells (Geuens et al., 2003, Nakatani et al., 2004) without clearly characterizing the specific cell type. However, discriminating the localization of two transcript variants at protein level might be very challenging, especially because the isoforms display the same amino acid sequence, as for CYGB and CYGB-A1. To overcome this difficulty, *in situ* hybridization-based experiments using specific probes targeting the canonical or the alternative first exon could be performed in different tissues and at different stages of development.

Final remarks

In conclusion, the results of this thesis contribute to the understanding of CYGB function using a novel cell model. We demonstrated that CYGB protects podocytes from oxidative stress and cell death, consistent with previous reports. Additionally, the anti-oxidative function of CYGB could be

also mediated by an alternative CYGB transcript variant that displays different transcriptional regulation. We suggested that CYGB may be associated with CKD, particularly with DN, which is currently under investigation using *in vivo* models.

Our study provides the basis for future investigations to unravel the role of CYGB in kidney disease. The association between CYGB and CKD represents an unexpected finding that, if validated, would contribute to an improved understanding of how an oxygen-binding globin can take part in the complexity of oxygen and ROS signaling in the diseased kidney.

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6. Contributions to the thesis

Publication mentioned in this thesis

1. Elisa B. Randi, Maria Tsachaki, Elena Porto, Maja T. Lindenmeyer, Clemens D. Cohen, Thomas Hankeln, Olivier Devuyst, Alex Odermatt, Roland H. Wenger and David Hoogewijs (2016). The anti-oxidative role of cytoglobin in podocytes suggests a potential association with chronic kidney disease. **In preparation.**

All figures.

2. Elisa B. Randi, Elena Porto, Thomas Hankeln, Roland H. Wenger and David Hoogewijs (2016). Identification of an alternative cytoglobin transcript variant lacking the A and B helices**. Unpublished results.**

All figures.

Publications not mentioned in this thesis

3. Katrin Gutsche, **Elisa B. Randi**, Volker Blank, Daniel Fink, Roland H. Wenger, Cornelia Leo, Carsten C. Scholz (2016). Intermittent hypoxia confers pro-metastatic gene expression selectively through NF-κB in inflammatory breast cancer cells. **Free Radic Biol Med**,101:129-142.

Immunoblot for Figure 7 C-E

Supplementary Figure S6

Quantification cell death Figure R2C (rebuttal letter)

7. Curriculum Vitae

Surname: RANDI

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- High School Diploma at Liceo Scientifico “G. Ferraris”, Varese, Italy. Year of graduation: 2006. Mark:74/100

- Bachelor degree in Biological sciences (cellular-molecular biology) at University of Insubria, Varese, Italy. Length of graduate studies: 3 years. Year of graduation: 2009. Mark: 104/110.

- Master Degree in Biological sciences (biomedical research) at University of Insubria, Varese, Italy. Length of graduate studies: 2 years. Year of graduation: 2010. Mark: 110/110, *cum laude*. Title of diploma thesis: *“Transcriptional gene inhibition by small RNA: effects of chemical modifications on silencing efficiency”.* Subject: Molecular Oncology. Institute of Oncology Research (IOR) in Bellinzona, Switzerland.

- Short term research attachment at the Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore, Singapore. Project title: *“Epigenetic variations during DNA hypomethylation”.*

- Employed as a PhD student in Prof. Roland Wenger’s lab (Institute of Physiology, University of Zurich) since 12th of November, 2012.

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